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THE MECHANISM OF THE ANTI-BACTERIAL ACTION OF MERCURY.

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STAMP (1939) and Woods (1940) have each independently studied the mode of action of sulphanilamide from the point of view that the action might be due to an interference with some " growth factor " essential for the organism.* This theory was first referred to by McIntosh and Whitby (1939).

It is now recognized that the series of reactions which are associated with the life of a cell, as evidenced by growth, are catalysed by a number of different chemical substances. These are often synthesized by the cells themselves but, in cases where they are not, they are recognized as " growth factors " which must be supplied in the nutrients or in some cases, in animal nutrition, as " vitamins."

It is also established that certain enzyme reactions can be specifically poisoned by various reagents in a manner which is not clearly understood, and it seemed not improbable that the growth-inhibiting effect of antiseptics or anti-bacterial substances in general might also be due to a specific poisoning of some essential reaction exerted through an interference with the catalyst or essential metabolite.† This interference might take place in more than one way. For instance the growth inhibitor might combine with the

* In a very recent paper Green (1940) concludes that sulphanilamide inhibits an enzymic reaction in the bacterial cell, which is stimulated by a " P " factor present in the cell and exerting a fundamental effect in metabolism. He considers that his " P " factor " is not an accessory growth factor in the usual meaning of this term."

† This term is used in preference to " essential growth factor " as the latter has been associated with essential nutrients. The " essential metabolite " is an organic substance without which metabolism cannot proceed to the extent required by growth. It is not necessarily a " growth factor " which must be provided in the nutrients. The term does not cover sources of energy, which are not specifically essential.

essential metabolite and form an inert product or, on the other hand, it might compete with the metabolite for the enzyme involved. In either case it might be possible to show some quantitative relationship between the inhibitor and the metabolite.

The idea of interference with an essential metabolite by an inhibiting agent was illustrated by Fildes in 1936 (1937) in connection with the bacteriostatic action of a diffusible O/R dye. This action was ascribed to the oxidation of some compound (essential metabolite) within the cell, which required to be reduced in growth. In the present paper the anti-bacterial action of mercury is considered from the same point of view.

It is well known that a diffusible mercury salt in small concentration has a high anti-bacterial action, and that this action can be prevented or antagonized within limits by H_2S. It is generally assumed, and no doubt correctly, that the action of H_2S is to form an insoluble sulphide and thus remove the Hg from its sphere of action. On the other hand, the anti-mercurial action of H–SH is also exerted by other –SH compounds which do not form insoluble compounds with mercury, but soluble compounds which, however, do not contain the –SH group. The addition of a diffusible Hg salt to a bacterial suspension will thus lead to the disappearance of intracellular –SH groups. Now the compounds R–SH are essential metabolites for many, if not all, cells, for instance for moulds (Volkonsky, 1932, 1933, 1934) and for staphylococcus (Fildes and Richardson, 1937), and without these groups within the cell, growth cannot take place. Thus the anti-bacterial action of Hg might be ascribed to its interference with the essential metabolite R–SH.

EXPERIMENTAL.

The experimental data in support of this view are as follows:

Bact. coli was exposed to the action of Hg and Hg-antagonistic substances in liquid cultures. The medium used was the ammonium lactate medium used by Fildes (1938) modified as follows: The Mg and Fe were added to the bulk before sterilization. The medium was then boiled for 5 min., cooled, filtered, distributed and autoclaved.

The experimental mixtures (10 ml. volume) were made in test tubes and the inoculated tubes then poured into 50-ml. Erlenmeyer flasks and incubated in air + 5 per cent. CO_2. In all cases the inoculum consisted of 1000 organisms from a previous ammonium lactate culture, the dilutions being made in the sterile medium.

In a preliminary experiment falling concentrations of $HgCl_2$ to $4 \times 10^{-8} M$ all completely inhibited growth. In the control a "trace" of growth was seen in 20 hours and full growth in 3 days. This is the usual rate of growth under these conditions.

The Anti-mercurial Action of Sulphur Compounds.

In Experiment 1 the effects of substances containing sulphur were tested. It will be seen that the action of Hg is neutralized by three –SH compounds but not by the –SS compounds nor by methionine.

Experiment 1 : *Anti-mercurial effect of sulphur compounds.*

$HgCl_2$, 8×10^{-5} *M*,was autoclaved in water and 1 ml. added per tube. Thiolacetic acid, 10^{-1} *M* in *N*/1 HCl, was autoclaved, neutralized and diluted 10^{-2} *M*. Glutathione and cysteine HCl were filtered as 10^{-2} *M* solutions. Cystine was 5×10^{-2} *M* (as S) in *N*/10 HCl, filtered and diluted to 10^{-2} *M*. Methionine, 10^{-2} *M*, was filtered. The inoculum was added last.

Flask.		Hg. (8×10^{-6} *M*.)	S. compounds.	($\times 10^{-6}$ *M*.)	Growth in hours and days. 21.	44.	3.
1	Medium	0	..	..	tr.	++	+++
2	,,	+	..	..	0	0	0
3	,,	+	thiolacetate	1000	tr.	++	+++
4	,,	+	..	200	?	+	+++
5	,,	+	..	40	0	0	0
6	,,	+	cysteine	1000	tr.	++	+++
7	,,	+	..	200	tr.	+	++
8	,,	+	..	40	0	0	0
9	,,	+	glutathione	1000	tr.	++	+++
10	,,	+	..	200	tr.	++	+++
11	,,	+	..	40	?	+	++
12	,,	+	cystine	1000	0	0	0
13	,,	+	..	200	0	0	0
14	,,	+	..	40	0	0	0
15	,,	+	methionine	1000	0	0	0
16	,,	+	..	200	0	0	0
17	,,	+	..	40	0	0	0

Plus signs are roughly proportional to the mass of growth. tr. = trace ; ? = doubtful.

Specificity of the Action of –SH.

In Experiment 2, carried out in the same way, it was shown that the following essential metabolites had no action antagonistic to Hg in the concentrations used, either singly or in various combinations, although growth was sometimes stimulated as compared with the control in the absence of Hg :

Aneurin (10^{-6} *M*), nicotinamide (2×10^{-6} *M*), riboflavine (10^{-6} *M*), vitamin B_6 (10^{-4} *M*), pantothenic acid (10^{-6} *M*), uracil (5×10^{-6} *M*), pimelic acid (2×10^{-5} *M*), β alanine (3×10^{-6} *M*), ascorbic acid (10^{-3} *M*), glutamine (10^{-3} *M*), and tryptophane (10^{-3} *M*).

Neither had glucose any action, although growth was much increased in rapidity. It is, therefore, concluded that the antagonistic action of –SH is specific.

Combining Proportions of Hg and –SH.

When a solution of an –SH compound is mixed with an excess of $HgCl_2$ and the mixture immediately tested for free –SH by the nitroprusside test, it is found that no –SH is present. This reaction carried out quantitatively may be used to determine the combining proportions of the two substances.

It is seen (Exp. 3) that both in the case of glutathione and of thiolacetic acid, 1 mol. Hg combines with 2 mol. –SH. This being so it is easy to understand the great toxicity of $HgCl_2$ under the present conditions. Since no –SH is in the medium, it is necessary to add only sufficient Hg to combine with the molecules of –SH present in 1000 organisms.

Experiment 3 : *Combining proportions of Hg and –SH compounds.*

Glutathione and thiolacetic acid (neutralized) were made up as 2×10^{-3} *M* solutions and the final concentration in the test was 2×10^{-4} *M*. $HgCl_2$ was 4×10^{-4} *M*. Falling quantities were added to give a final concentration from 2×10^{-4} *M* downwards.

The mixtures were made, and a nitroprusside test carried out immediately. The limit of this test was approximately 10^{-5} *M*.

Tube.	Water. (ml.)	–SH. (ml.) ($\equiv 20 \times 10^{-5}$ *M* final.)	Hg. ml.	Hg. ($\times 10^{-5}$ *M*.)	Nitroprusside test.
1	9·0	+ 1·0	0	..	+++
2	4·0	+ 1·0	5·0	20	0
3	6·5	+ 1·0	2·5	10	0
4	7·33	+ 1·0	1·66	6·6	tr.
5	7·75	+ 1·0	1·25	5	+
6	8·0	+ 1·0	1·0	4	++

Quantitative Relationships between Hg and –SH in Growth.

In Experiment 1 it is seen that the action of glutathione antagonistic to Hg is greater than that of thiolacetic acid on a molecular basis. This is contrary to expectation if the antagonistic action is due solely to –SH, since the combining proportions of the two substances are equal. Experiments 4 and 5 show quantitatively the effects in growth. Experiment 4 refers to glutathione. It shows that the concentration of –SH must be four times that of Hg to allow growth. On other occasions three times the concentration has been sufficient, but never merely twice the concentration. Experiment 5 refers to thiolacetic acid. In this case 25 times the concentration of –SH is necessary, while in the smallest concentration tested (10^{-4} *M*) even this is not enough. It will be understood that since –SH compounds are subject to oxidation during storage and sterilization of the solutions, the supposed molarity of a solution must necessarily be less at the time of the experiment and, therefore, 2 mol. of the solution will not be sufficient completely to combine 1 mol. Hg. Since mere traces of excess Hg will inhibit growth, it is necessary, in practice always to have some excess of –SH present. In the case of glutathione 3–4 mols is sufficient, instead of the theoretical 2, but in the case of thiolacetic acid much more is necessary. This difference between the two –SH compounds cannot be explained by a greater lability of –SH in thiolacetate. Thiolacetic acid in HCl is much more stable than glutathione in water and it is also more stable under the conditions of the culture.

Further investigations into the combination of these compounds with Hg were undertaken to explain this phenomenon.

Experiment 4: *Quantitative relationship between glutathione and Hg in growth.*

Details as in Experiment 1.

Flask.		–SH. ($\times 10^{-5}$ *M.*)	Hg. ($\times 10^{-5}$ *M.*)	Growth in hours and days. 24.	43.	3.	4.
1	Medium	+	0	0	+	+++	+++	+++
2	,,	+	20	20	0	0	0	0
3	,,	+	20	10	0	0	0	0
4	,,	+	20	5	0	+	++	+++
5	,,	+	20	2·5	tr.	++	+++	+++
6	,,	+	10	10	0	0	0	0
7	,,	+	10	5	0	0	0	0
8	,,	+	10	2·5	0	++	+++	+++

Experiment 5: *Quantitative relationship between thiolacetate and Hg in growth.*

Details as in Experiment 1.

Flask.		–SH. ($\times 10^{-6}$ *M.*)	Hg. ($\times 10^{-6}$ *M.*)	Ratio: Hg/–SH.	Growth in 2 days.
1	Medium	+	0	0	..	+++
2	,,	+	800	64	1/12·5	0
3	,,	+	,,	32	1/25	+++
4	,,	+	,,	16	1/50	+++
5	,,	+	,,	8	1/100	+++
6	,,	+	400	32	1/12·5	0
7	,,	+	,,	16	1/25	+++
8	,,	+	,,	8	1/50	+++
9	,,	+	,,	4	1/100	+++
10	,,	+	200	32	1/6·25	0
11	,,	+	,,	16	1/12·5	0
12	,,	+	,,	8	1/25	+++
13	,,	+	,,	4	1/50	+++
14	,,	+	100	16	1/6·25	0
15	,,	+	,,	8	1/12·5	0
16	,,	+	,,	4	1/25	0

Differences in the Behaviour of Glutathione and Thiolacetate.

When $HgCl_2$ in sufficient concentration is added to the ammonium lactate medium a precipitate is formed. Analysis of the reagents involved shows that the precipitate depends on the presence of NH_4Cl and $HgCl_2$ at a slightly alkaline pH, and it is presumed to be one of the mercury-ammonium chlorides. This precipitation is prevented, or the precipitate is re-dissolved, by sufficient –SH, and when the precipitate persists in the presence of –SH it is concluded that Hg exists in the mixture uncombined with –SH but combined with NH_3.

Experiment 6 shows that the effectiveness, as a solvent of the precipitate, of glutathione is greater than that of thiolacetate. Some four times the concentration of the latter is necessary to give the same result as the former, i.e. to effect a complete combination of the Hg with –SH. Under the conditions of a cultivation the concentration of Hg is too small to produce a precipitate, but it is supposed that the mercury-ammonium compound is in solution, and capable of producing the toxic effect of Hg by combination with the –SH of the organism. Before the mercury-containing medium can be rendered adequate for growth, sufficient –SH must be added to effect complete combination with the Hg in the compound, and Experiment 6 indicates that more thiolacetate is necessary for this purpose than glutathione. The difference between glutathione and thiolacetate in a growth test is thus ascribed to differences in chemical behaviour rather than to differences in the action performed.

Experiment 6 : *Effect of –SH on the production of the Hg-NH_3 precipitate.*

Thiolacetate $1{\cdot}6 \times 10^{-1}$ *M* was diluted 1/10 with the medium. Glutathione 2×10^{-2} *M* was similarly diluted. A series of 1/2 further dilutions were made with the medium and 1 ml. of each mixed with 1 ml. 5×10^{-3} *M* $HgCl_2$.

Tube.				($\times 10^{-4}$ *M.*)		Ppt.
1	1 ml. medium	+	thiolacetate	160	+ Hg	0
2	,,	+	,,	80	+ ,,	0
3	,,	+	,,	40	+ ,,	±
4	,,	+	,,	20	+ ,,	+
5	,,	+	,,	10	+ ,,	|
6	,,	+	,,	5	+ ,,	|
7	,,	+	,,	2·5	+ ,,	↓
8	,,	+	,,	1·25	+ ,,	++
9	,,	+	glutathione	20	+ ,,	0
10	,,	+	,,	10	+ ,,	tr.
11	,,	+	,,	5	+ ,,	±
12	,,	+	,,	2·5	+ ,,	+
13	,,	+	,,	1·25	+ ,,	++
14	,,	..	..		+ ,,	+++

The Reversibility of the Anti-bacterial Action of Hg.

In these experiments the various mixtures were made in the test-tubes before the addition of the test bacteria. The combination between Hg and –SH had therefore taken place before inoculation, and the inoculum was exposed to such excesses of Hg or –SH as there might be. Since these two compounds are diffusible and the mass of bacteria so small, it is assumed that their relative concentrations in the medium are a true index of their concentrations in the bacteria. When the bacteria are added to the Hg before the –SH is added, the concentration of the former in the cell is, of course, greater, but the effect of –SH subsequently added is practically the same.

There is a little delay in the rate of growth but quantitatively the Hg/–SH ratios are not materially altered. Even when the bacteria were exposed for four days at room temperature to 2×10^{-7} *M* $HgCl_2$, the addition of excess –SH allowed growth without obvious delay. On the other hand, bacteria exposed to 2×10^{-6} *M* Hg at 38° for 17 hours did not grow on adding –SH. Within limits, therefore, which have not been determined accurately, the anti-bacterial action of Hg is reversible by –SH, as is well known to be the case with H_2S. It is concluded that, within limits, the action of Hg is merely to inactivate the –SH group without other demonstrable injury to the cell.

CONCLUSIONS.

1. The anti-bacterial action of Hg is specifically neutralized by –SH compounds.
2. In the case of glutathione this neutralization takes place approximately in the proportions 1 mol. Hg/2 mol. –SH.
3. Glutathione combines with Hg in these proportions to form a compound devoid of –SH.
4. Hg combines with –SH in the cell to form a similar compound and thus deprives the cell of –SH groups which are essential for its metabolism.
5. These findings accord with a view that anti-bacterial substances may operate by interfering with essential metabolites in a cell.

I have to thank Prof. James McIntosh, in whose institute this work was carried out, and Dr. L. E. H. Whitby for information relating to the behaviour of sulphanilamide, and my colleagues, Dr. D. D. Woods and Dr. H. McIlwain, for criticism during the course of the work.

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THE RELATION OF *p*-AMINOBENZOIC ACID TO THE MECHANISM OF THE ACTION OF SULPHANILAMIDE.

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In the preceding paper Fildes (1940) discusses a general hypothesis that disinfectants and other substances preventing the growth of bacteria act by interfering in some way with substances essential to the growth of the organism. In the present work the mode of action of sulphanilamide is investigated from the point of view of this general hypothesis.

A large number of substances known to be associated with bacterial metabolism were tested to determine whether they had an antagonistic relation to sulphanilamide akin to that of –SH and Hg but without conclusive results. While this work was in progress, Stamp (1939), working on a similar hypothesis, found that extracts of streptococci were able to antagonize the action of sulphanilamide and later, while the present paper was actually in preparation, Green (1940) obtained a preparation from *Brucella abortus*. Following Stamp's procedure it was found that yeast extracts contained a substance which, like that of Stamp and Green, reversed the inhibitory action of sulphanilamide. The chemical properties of this substance and its behaviour in growth tests indicated that it might be chemically related to sulphanilamide itself. As a result of this suggestion *p*-aminobenzoic acid was tested and found to have high anti-sulphanilamide activity. A preliminary report of this work has already been given (Woods and Fildes, 1940). The bearing of these results on the possible mode of action of the drug is discussed.

BACTERIOLOGICAL TECHNIQUE.

The organism used was *Streptococcus haemolyticus* (Richards). Stock cultures were maintained on meat infusion peptone agar and were passaged through mice every 14 days.

Medium.

The test medium was buffered Bacto-peptone (McIlwain, Fildes, Gladstone and Knight, 1939), tubed in 7 ml. lots and autoclaved, with the addition of the following materials to each tube at the time of testing :

* Halley Stewart Fellow.

	ml.
Phosphate buffer $M/18$, pH 7·6	1·175
List A (in $N/5$ HCl)	0·5
N NaOH	0·1
$M/50$ glutamine	0·125
Cocci (in peptone water)	0·1
Sulphanilamide $M/300$, or water	1·0
Test solution, or water	1·0

List A contained glucose, KH_2PO_4, aneurin, nicotinamide, β-alanine, pimelic acid and riboflavin in quantities given by McIlwain *et al.* (1939) and also 0·24 g. cystine/100 ml. The mixture was made up, stored and sterilized (by filtration) in $N/5$ HCl. The total volume of the test medium was thus 11 ml. and the final concentration of sulphanilamide $M/3300$.

At the time this work was begun the above medium was one of the simplest known on which the streptococcus will grow satisfactorily (McIlwain *et al.*, 1939). It was felt desirable to use as simple a medium as possible, as it is known that the inhibitory action of sulphanilamide is less complete in complex media containing, for example, serum or meat broth.

Inoculum.

For satisfactory testing it was necessary that sulphanilamide inhibition should be as complete as possible, and a standard of no growth for at least five days with $M/3300$ sulphanilamide was aimed at. To attain this it was found that both the size and age of the inoculum were of importance (Table I). Young cultures and large inocula both reduced the inhibitory power of the drug. As a routine therefore the inoculum chosen was *ca.* 1000 cells from a two or three day stock culture. It was also found that if the meat infusion broth used for the stock culture medium was freshly prepared inhibition was again unsatisfactory; freshly-made medium was therefore avoided. With these precautions inhibition was complete, with few exceptions, for the duration of the test (five to six days).

TABLE I.—*Effect of Size and Age of Inoculum on Sulphanilamide Inhibition.*

Cultures on meat infusion peptone agar used as source of inocula.
All tubes contained $3·03 \times 10^{-4}$ M sulphanilamide.

Age of agar cultures (hrs.).	Number of cells inoculated.	Growth after days. 1.	2.	3.	5.
24	10^6	+	+++	+++	+++
	10^5	0	+++	+++	+++
	10^4	0	0	+++	+++
	10^3	0	0	0	0
48	10^6	+	+++	+++	+++
	10^5	0	++	+++	+++
	10^4	0	0	0	0
	10^3	0	0	0	0

Here and elsewhere + signs are roughly proportional to the mass of growth. Above experiment repeated in absence of sulphanilamide gave +++ in all cases in one day.

Tests.

Activity in antagonizing the inhibitory effect of sulphanilamide was followed by determining the minimum amount of material necessary to promote full growth in the presence of a standard concentration ($M/3300$) of the drug. In each test the following series of tubes were set up: (1) medium alone (full growth normally attained in one day or less), (2) medium + sulphanilamide (in duplicate), (3) medium + top concentrations of test materials used (to test for any inhibitory or growth-stimulating effect), (4) medium + sulphanilamide + test material in falling concentration from a 1 in 5 serial dilution. Tubes were incubated in air + 5 per cent. CO_2 at 37° C. and readings taken daily for five days. It was observed throughout that if growth occurred at all, it always became maximal within 24 hours of the appearance of the first trace.

ANTI-SULPHANILAMIDE ACTIVITY OF CELL EXTRACTS, ETC.

The failure to obtain any significant activity with known essential substances led to a consideration of the possibility that the substance postulated might be one whose importance in cell metabolism had not yet been recognized and a survey was made of cell extracts, etc. At this point Stamp (1939) demonstrated that extracts of streptococcal cells contained a substance which powerfully antagonized the inhibitory action of sulphanilamide and the related drug M. & B. 693. As Stamp points out, this substance, which appears normally to be present in the cell, may be the metabolite with which the action of sulphanilamide is concerned. By applying Stamp's method of extraction (incubation in $N/25$ NH_3 at 37° C.) to baker's yeast we were able to obtain

TABLE II.—*Anti-Sulphanilamide Activity of Cell Extracts, etc.*

Activity expressed as minimum volume required to give growth in $3{\cdot}03 \times 10^{-4}$ M sulphanilamide.

Material.	Method of extraction.	Concentration (g./ml. extract).	Activity.
Baker's yeast	Stamp	3·5	0·0016
Yeast wash	..	≡3·5	0·0016
Brewer's yeast	Stamp	3·5	0·04
,, ,,	H_2O, 100° C.	3·5	0·04
,, ,,	Na_2SO_4*	3·5	1·0
Ox muscle	H_2O, 100° C.	1·0	0·2
,, ,,	Tryptic digest	1·7	0·2
,, liver	H_2O, 100° C.	1·0	0·2
Turnip	,,	1·7	0·2
Urine	..	..	0·04
Serum (horse)	..	..	0·04
Laked blood, 50 per cent. (ox)	..	..	0·2
Albumin (egg)	HCl hydrolysed	0·1	<1·0

* Method of Deutch, Eggleton and Eggleton (1938).

more active preparations than by other methods. Such extracts were also more active than the other cell extracts tried (Table II), and seemed to be a suitable starting-point for larger scale work. Less active extracts were obtained if the yeast was washed prior to extraction, and it seemed possible that a greater amount of factor might be obtained from the medium on which the yeast had been grown. A sample of yeast wash* was tested (after precipitation of inactive material with alcohol), but was found to have no higher activity (on basis of weight of yeast obtained from it) than the yeast extracts. It also contained a greater bulk of inactive material.

FRACTIONATION AND CHEMICAL PROPERTIES OF YEAST EXTRACT.

Preparation of Yeast Extract.

The procedure of Stamp for streptococci was modified to some extent for experimental convenience; in particular the incubation time was reduced from 24 to 5 hours, as this resulted in no decrease of activity and there was less trouble with contamination by anaerobes.

7 lb. fresh baker's yeast (D.C.L.) were crumbled into 5250 ml. $N/25$ NH_3 previously warmed to 43° C. and stirred till evenly suspended; the pH was then *ca.* 7·8. The suspension was incubated five to six hours at 37° C. with occasional shaking; the pH normally fell to 5·5–6. The bulk of yeast was removed by filtration through pulp, and the remainder with the Sharples centrifuge. The clear supernatant (5300 ml.) was evaporated *in vacuo* to 200 ml. and 9 volumes of alcohol added. The ppt. (inactive) was discarded. The filtrate was taken to dryness *in vacuo* and yielded *ca.* 20 g. of a thick brown syrup which was taken up in water, adjusted to pH 7, diluted to 180 ml., and filtered from a small amount of insoluble material. This solution will be referred to as Extract A. For testing it was diluted 1 in 5 to a concentration of 3·5 g. original yeast/ml. (referred to throughout as "standard strength"). Extract A is thus 5 × "standard strength." The average anti-sulphanilamide activity of several batches of Extract A was *ca.* 30 μg. dry wt./11 ml. medium (i.e. 3 parts per 10^6 medium).

Preliminary Purification of Extract.

The active material was precipitated by mercuric acetate but not by phosphotungstic acid (Table III). The latter reagent precipitated some inactive material, and a combination of the two methods removed most of the colour and reduced the dry weight of material by 50 per cent. without serious loss of activity.

Mercury precipitation.—160 ml. Extract A were treated with an excess of mercuric acetate (130 ml. 30 per cent.). The mixture was kept overnight at 0° C. and filtered. The ppt. was washed twice by resuspension in 50 ml. water containing a little mercuric acetate, decomposed with H_2S and filtered. The filtrate and two washings of the HgS ppt. were combined, aerated to remove

* I am indebted to British Fermentation Products, Ltd., for the gift of this material.

H_2S, neutralized, and evaporated to 160 ml. The original Hg filtrate was worked up in the same way for testing.

Phosphotungstic acid precipitation.—160 ml. of the decomposed Hg ppt. (as above) were acidified with 40 ml. 5*N* H_2SO_4 and an excess of 20 per cent. phosphotungstic acid in *N* H_2SO_4 added (130 ml.). After 4½ hours at 0° C. the ppt. was filtered off and washed twice by resuspension in *N* H_2SO_4 containing 1 per cent. phosphotungstic acid. Filtrate and washings were treated with $Ba(OH)_2$ until no further ppt. was obtained, and excess of $Ba(OH)_2$ was present. The filtrate (and two washings of the Ba ppt.) were slightly acidified with H_2SO_4 to remove excess Ba and refiltered. The filtrate was neutralized and evaporated *in vacuo* to 160 ml. This solution will be referred to as Extract B and is again equivalent to 5 × "standard strength."

The original phosphotungstic ppt. was decomposed by solution in warm acetone and treating with $Ba(OH)_2$. It was also tested.

The dry wt. of Extract B was *ca.* 50 mg./ml., of which 25 mg. were ash. The average activity was 16 μg./11 ml. or 1·5 parts/10^6 medium. Extract B was used for most of the following work.

Chemical Properties of the Factor.

(a) *Stability.*

The activities of Extracts A and B were unaffected by boiling, autoclaving at 15 lb. for 15 min. or heating to 100° C. for 30 min. with 0·2 *N* HCl or NaOH. It was also stable to boiling for some hours with 50 per cent. HCl. The extracts did not lose activity on storage at 0° C. for three months.

TABLE III.—*Precipitation of Factor with Metals, etc.*

Activity is expressed as the reciprocal of the minimum volume of test solution of "standard strength" (i.e. ≡ 3·5 g. original yeast/ml.) required to give growth with 3·03 × 10^{-4} *M* sulphanilamide.

Reagent.	Activity. Original solution.	Ppt.	Filtr.	Ratio: ppt./filtr.
Mercuric acetate	625	625	25	25/1
,, ,,	625	625	25	25/1
Phosphotungstic acid	625	25	625	1/25
,, ,,	125	5	125	1/25
Basic lead acetate	625	125	625	1/5
Silver nitrate, pH 2	625	5	625	1/125
,, ,, pH 7	625	625	125	5/1
,, ,, ,,	625	625	25	25/1
,, ,, ,,	125	25	25	1/1

(b) *Precipitation by metals, etc.*

Results are given in Table III. Precipitation with Hg and non-precipitation with phosphotungstic acid were clear cut and consistently obtained,

and were therefore useful in routine purification. Ag at pH 7 gave variable results; the precipitation appeared to be less complete with weaker preparations. The separation with basic lead acetate was not sufficiently distinct for practical use.

(c) *Solvent extraction.*

The active material was extracted by ether from weakly acid (pH 4·5), but not from weakly alkaline (pH 8·5) solution (Table IV). The substance was completely recovered from ethereal solution by extraction with 1 per cent. Na_2CO_3. With butyl alcohol the factor was extracted completely at pH 3·5, and only partially at pH 7. These results indicate that the factor has weak acidic properties. The factor was extracted from weak acid solution by ethyl acetate but not by petrol ether.

TABLE IV.—*Extraction of Factor from Aqueous Solution with Various Solvents.*

Activity expressed as in Table III. The aqueous solution was extracted 5 times with an equal volume of solvent.

Solvent.	pH of aqueous phase.	Activity. Original solution.	Extract.	Residue.	Ratio: Extract/residue.
Ether	2·0	625	125	<1	>125/1
,,	4·5	625	625	5	125/1
,,	8·5	625	<1	625	<1/125
Butyl alcohol	3·5	625	625	5	125/1
,, ,,	7·0	625	125	125	1/1
Ethyl acetate	4·0	125	125	1	125/1
Petrol ether	4·0	125	<1	125	<1/125

(d) *Esterification.*

The presence of an acidic group was confirmed by the formation of an ethyl ester. The activity of the esterified material was very slight, but on hydrolysis the activity of the original extract was recovered (Table V). 20 ml. Extract B was brought to pH 4 and extracted with ether to obtain the free acid. The dried material was refluxed four hours with 1 per cent. HCl in ethyl alcohol. Alcohol and HCl were removed, the residue taken up in 20 ml. water and a 4 ml. sample of this esterified material removed for testing. The remainder was brought to pH 8·5 with $NaHCO_3$ and esters separated from any unchanged acid by ether extraction. The ether extract was evaporated, the residue hydrolysed by boiling 1½ hours with 50 per cent. (by vol.) HCl, taken to dryness, dissolved in water, neutralized and tested.

(e) *Treatment with nitrous acid.*

Activity was almost completely destroyed by mild treatment with HNO_2 in dilute aqueous solution, and this result suggests that the factor may also contain an amino group (Table V). 45 mg. barium nitrite was added to 2 ml.

Extract B at 0° C. and the mixture brought to pH 3 with H_2SO_4; after standing overnight at 0° C. it was brought to room temperature and finally to 37° C. for an hour. The solution became orange. Excess HNO_2 was removed by urea (22 mg.) and $BaSO_4$ centrifuged off.

TABLE V.—*Other Chemical Properties of the Factor.*

Activity expressed as in Table III.

Treatment.	Activity. Original material.	Product.
Nitrous acid	625	5
Acetylation	125	<1
Hydrolysis of acetylated product	..	125
Ethyl esterification	125	5
Hydrolysis of esterified product	..	125
Norite adsorption	625	1
Combined Na_2CO_3, alcohol, and alcohol-pyridine eluates of norite	..	5
Steam distillation	125	<1

(f) *Acetylation.*

Confirmatory evidence for the presence of an amino group was obtained from the observation that acetylation of the extracts led to loss of activity, and that the original activity was regenerated by hydrolysis of the acetylated product (Table V). These results might also, of course, indicate the presence of an OH group. 20 ml. Extract B were taken to dryness and dried over P_2O_5. 3 ml. pyridine and 2 ml. acetic anhydride were added, the mixture heated to 100° C. for 10 hours and then taken to dryness. Traces of pyridine were removed by dissolving in alcohol and re-evaporating. The acetylated material was taken up in water, neutralized, diluted to 20 ml. and 10 ml. taken for testing. The remainder was hydrolysed by saturating with HCl and boiling for 1½ hours.

(g) *Other properties.*

The active material was strongly adsorbed by norite charcoal (0·3 g./3 ml. Extract B). Elution with 1 per cent. Na_2CO_3, acid-alcohol and 10 per cent. pyridine in alcohol recovered only traces of the factor (Table V). The factor was not volatile in steam from acid solution. An alcohol extract of dried Extract A had equal activity to the original solution.

Survey of Chemical Properties.

The factor has weakly acidic properties [(c) and (d)]. The acid pK (*ca.* 4–5) indicated by the extraction experiments is strong evidence of the carboxylic nature of the acidic group, and is suggestive of unsubstituted fatty acids and aromatic carboxylic acids. The additional presence of an amino group [(e) and (f)] indicates that the factor is an ampholyte. The solubility in ether

excludes, in general, any ampholyte that exists in solution as a zwitterion (e.g. aliphatic or aliphatic side-chain amino acids). This in conjunction with the relatively weak acid dissociation suggested that the factor might be of the type of an amino derivative of an aromatic carboxylic acid. Purified factor preparations (acid ether extract and hydrolysed esterified material) produced a red colour on diazotizing and coupling with dimethyl-α-napthylamine; this confirms the presence of a substance containing an aromatic amino group, but does not prove that this substance is the active substance. If the factor is indeed an amino derivative of an aromatic carboxylic acid (there are, of course, other possibilities), it would bear some chemical relationship to sulphanilamide itself. This will be discussed further in the next section in connection with evidence of a different type tending to the same conclusion.

PROPERTIES OF THE FACTOR IN GROWTH TESTS.

Concurrently with the chemical fractionation some experiments on the possible mode of action of the anti-sulphanilamide factor were carried out with partly purified material.

TABLE VI.—*Titration of Anti-Sulphanilamide Activity of Yeast Extract A.*

	ml. extract A.	Growth after hours and days. 18.	42.	3.	5.
Medium alone	.	+	+++	+++	+++
,, + sulphan. (duplicate)	.	0	0	0	0
,, 0 + Extract A	1·0	+++	+++	+++	+++
,, 0 + ,,	0·2	++	+++	+++	+++
,, + ,, + ,,	1·0	+++	+++	+++	+++
,, + ,, + ,,	0·2	+++	+++	+++	+++
,, + ,, + ,,	0·04	tr.	+++	+++	+++
,, + ,, + ,,	0·008	0	tr.	+++	+++
,, + ,, + ,,	0·0016	0	0	+++	+++
,, + ,, + ,,	0·0003	0	0	0	0

Table VI shows the results of a typical titration for determination of activity. The following points may be noted:

(1) The extract has some growth-promoting activity at the higher concentrations. This type of activity became reduced with increasing fractionation, although anti-sulphanilamide activity remained unchanged.

(2) The bacteriostatic nature of sulphanilamide inhibition is confirmed. With the smaller amounts of factor the organism grew up after 2–3 days' incubation with sulphanilamide during which time no visible growth occurred.

(3) In the titration each tube contains 1/5 of the amount of factor in the preceding tube and this is the limit of accuracy of the method. If finer differences are used the results are less consistent.

It was soon evident from the sharpness of the chemical fractionation obtained with many of the precipitants and solvents used that the factor was probably a single substance and not a mixture of components each accelerating

growth. This is important, as it is known that vigorous growth, particularly during the early stages, diminishes the inhibitory effect of sulphanilamide in a non-specific manner.

It was first considered whether the factor might be working by reacting chemically with the sulphanilamide, and thus removing it from the medium. In this case it would be expected that the molar concentration of the factor would be of the same order as that of the sulphanilamide used. Comparison of the dry weight and activity of even crude factor preparations showed that this cannot be the case. 0·003 mg. of an acid ether extract of Extract B was sufficient to reverse the inhibitory action of 1 ml. 0·0033 *M* sulphanilamide. If the factor is equimolar with sulphanilamide, then 0·003 mg./ml. must be equivalent to 0·0033 *M* factor, and from this the mol. wt. of the factor works out at *ca.* 1. As even this result was obtained by assuming a crude preparation to be pure factor, it is certain that any molecular reaction can be excluded. It remained possible that the factor might act catalytically in promoting the removal of sulphanilamide.

In other experiments it was found that if the concentration of sulphanilamide is increased, it is necessary to raise the concentration of factor in the same proportion in order to reverse the inhibition. This constant quantitative relationship has been demonstrated with a number of different factor preparations at various stages of purification and also using *Bact. coli* in place of the streptococcus as the test organism. A selection of such experiments is given in Table VII. The fact that the concentration ratios are *exactly* constant is due to the fact that sulphanilamide and factor were each used in 1/5 falling concentration. As the titration cannot be used accurately with finer differences in amount of factor, it was considered better to carry out a number of separate titrations than to attempt to work with smaller differences. Sulphanilamide is insufficiently soluble for work with higher concentrations than those recorded and smaller amounts tend to give incomplete inhibition.

TABLE VII.—*Relation between Concentration of Sulphanilamide and Concentration of Factor Required to Reverse the Inhibition.*

Quantities of Extracts A and B are expressed as ml. " standard strength."

Organism.	Streptococcus.		*Bact. coli*.†		Streptococcus.	
Factor used:	Extract A.		Extract B.		*p*-aminobenzoic acid.	
Conc. sulphan. ($M \times 10^{-3}$). (*a*).	ml. required ($\times 10^{-1}$). (*b*).	Ratio:* (*b*) / (*a*).	ml. required ($\times 10^{-1}$). (*c*).	Ratio:* (*c*) / (*a*).	Conc. required ($M \times 10^{-7}$). (*d*).	Ratio: (*d*) / (*a*). ($\times 10^{-4}$).
0·303	0·016	5·3	0·08	26·5	0·58	1·92
1·515	0·08	5·3	0·4	26·5	2·91	1·92
7·575	0·4	5·3	2·0	26·5	14·54	1·92

* These arbitrary ratios are an expression of the ratio: conc. factor required/conc. sulphanilamide used.

† The strain of *Bact. coli* and the medium employed (lactate + inorganic salts) were those used by Fildes (1940).

As there was no possibility of a direct molecular reaction between sulphanilamide and the factor, this constant quantitative relationship between inhibitor

and active substance was reminiscent of the competitive inhibition of enzyme reactions by substances chemically related to the substrate or product. From this point of view the factor would be considered to be the substrate (or product) of the enzyme reaction in question and sulphanilamide the substance of related chemical structure inhibiting the reaction. It will be recalled that consideration of the chemical properties of the factor also indicated the possibility that the factor might be chemically related to sulphanilamide. Examples of known cases of competitive inhibition of enzyme reactions by substances related to the substrate or products are: (*a*) succinic dehydrogenase by malonic acid, (*b*) lipase (hydrolysis of ethyl butyrate) by acetophenone and other non-polar compounds containing a carbonyl group, and (*c*) invertase by α and β-galactose and β-*l*-arabinose. Other examples and full references to those quoted are given by Haldane (1930).

As it was possible to interpret these two distinct lines of evidence (chemical properties and behaviour in growth tests) in the same way, it seemed worth while at this stage to test for anti-sulphanilamide activity some compounds which are structurally related to sulphanilamide, and whose properties are reasonably in accord with those of the yeast factor.

ANTI-SULPHANILAMIDE ACTIVITY OF *p*-AMINOBENZOIC ACID.

In view of the probability that the acid group of the factor was carboxylic the first substance tested was *p*-aminobenzoic acid. In this acid the *p*-NH_2 of sulphanilamide is unchanged but $-SO_2NH_2$ is replaced by –COOH. *p*-aminobenzoic acid proved to have very high activity, and a final concentration of $1{\cdot}2–5{\cdot}8 \times 10^{-8}$ *M* was sufficient to reverse the inhibition caused by $3{\cdot}03 \times 10^{-4}$ *M* sulphanilamide (i.e. 0·02–0·1 μg. in 11 ml. medium compared with 570 μg. sulphanilamide). The results of a number of quantitative determinations are given in Table VIII. The agreement between separate determinations (maximum variation by a factor of 5) is satisfactory in view of the high dilutions used and possible differences in the state of the inoculum. Duplicates in the same determination did not show this variation.

TABLE VIII.—*Anti-sulphanilamide Activity of* p-*Aminobenzoic Acid.*

Sulphanilamide conc. $= 3{\cdot}03 \times 10^{-4}$ *M*.

Sample of *p*-aminobenzoic acid.	Commercial.	5 × recrystallized.	From acetyl derivative.	All.
Number of determinations:				
Total . .	7	5	3	15
Molarity				
Titrating to . $2{\cdot}91 \times 10^{-7}$.	0	1	0	1
,, . $5{\cdot}82 \times 10^{-8}$.	4	2	2	8
,, . $1{\cdot}16 \times 10^{-8}$.	3	2	1	6

Purity of p-*aminobenzoic acid.*

Table VIII also shows that the activity of *p*-aminobenzoic acid is unaffected by recrystallizing five times, or by acetylating and recovering the acid by

hydrolyzing the recrystallized acetyl derivative. The two purified specimens both melted at 186° C. uncorr. (literature, 186–7° C.) and gave the following analytical figures (Weiler, Oxford):—(*a*) 5 × crystallized: C, 61·38; H, 5·15; N, 10·55 per cent.; (*b*) acetylated and hydrolyzed: C, 61·48; H, 5·08; N, 10·52 per cent. (calculated: C, 61·31; H, 5·11; N, 10·22 per cent.). From the method of preparation the most likely impurities are the *o*- and *m*-isomers and *p*-nitrobenzoic acid; these were tested (Table IX) and found to be inactive, or very feebly active compared with *p*-aminobenzoic acid. (It is equally likely that the *m*-isomer is contaminated with traces of the active *p*-compound.) There is little doubt, therefore, that the active substance is *p*-aminobenzoic acid and not an impurity in the specimen used.

Quantitative relationship with sulphanilamide.

As with the yeast factor, there was a constant relationship between the concentration of sulphanilamide used and the concentration of *p*-aminobenzoic acid required to reverse the inhibition (Table VII). In order to avoid the variation in titration found in different experiments (see above), it is necessary that the titrations be carried out in the same experiment and with the same source of inoculum and serial dilution of factor. In a number of separate experiments with the same concentration of sulphanilamide (Table VIII) the ratio molar conc. sulphanilamide/molar conc. *p*-aminobenzoic acid required ranged from 5000–25,000, reflecting the usual factor of 5 variation.

Experiments with Bact. coli.

The action of *p*-aminobenzoic acid was also demonstrated with *Bact. coli* as test organism and an entirely synthetic basal medium of lactate and inorganic salts (Fildes, 1940). On this medium the usual amount of sulphanilamide ($3{\cdot}03 \times 10^{-4}$ *M*) inhibited completely for five days, and the inhibition was overcome by the same amount of *p*-aminobenzoic acid ($5{\cdot}8 \times 10^{-8}$ *M*) as was effective with the streptococcus. This was also the case when the activity of a particular yeast extract was measured with the two organisms.

Experiments with M. & B. 693.

Under the test conditions used in this work 2-(*p*-aminobenzenesulphonamido) pyridine (M. & B. 693) has approximately five times the inhibitory activity of sulphanilamide. *p*-Aminobenzoic acid also reverses inhibition by this drug, although five times as much is required as for a similar concentration of sulphanilamide. Similar results are obtained with the yeast factor.

	p-aminobenzoic acid ($M \times 10^{-3}$).	Extract A (ml. standard strength).
Sulphanilamide ($3{\cdot}03 \times 10^{-4}$ *M*)	5·8	0·0016
M. & B. 693 ,,	29·1	0·008

Sulphanilic acid ($0{\cdot}9 \times 10^{-3}$ *M*) also inhibits, though less efficiently, and this is again reversed by *p*-aminobenzoic acid.

Growth-promoting activity.

p-aminobenzoic acid had no significant effect on the rate or mass of growth under the test conditions. There was slight acceleration at high concentrations ($0{\cdot}2 \times 10^{-3}$ *M*), but above this there was slight inhibition. No growth stimulation was found with *Bact. coli* on the ammonium lactate medium which is certainly free from preformed *p*-aminobenzoic acid. The crude yeast extracts had considerable growth-accelerating action, but this was greatly diminished as the factor was purified. The anti-sulphanilamide factor (" P ") obtained from *Brucella abortus* by Green (1940) was reported to have high growth-promoting activity for a number of organisms, but the material had not at that time been much purified.

ANTI-SULPHANILAMIDE ACTIVITY OF SUBSTANCES RELATED TO *p*-AMINOBENZOIC ACID.

Materials.

p-*acetaminobenzoic acid* was prepared from *p*-aminobenzoic according to Ritsert and Epstein (1904). The product was washed with dil. HCl and recrystallized from 20 per cent. alcohol; M.P. 250° C. The *p*-aminobenzoic acid was regenerated by refluxing two hours with conc. HCl, taking to dryness, dissolving in water and bringing to pH 4·5 with sodium acetate. After three

TABLE IX.—*Anti-Sulphanilamide Activity of Substances Related to* p-*Aminobenzoic Acid.*

Conc. of sulphanilamide = $3{\cdot}03 \times 10^{-4}$ *M*.

Substance.	Active at *M* conc.
p-aminobenzoic acid	$1{\cdot}2$–$5{\cdot}8 \times 10^{-8}$
o-aminobenzoic acid	—
m-aminobenzoic acid	$0{\cdot}9 \times 10^{-3}$
p-nitrobenzoic acid	$1{\cdot}8 \times 10^{-4}$
p-acetaminobenzoic acid	$1{\cdot}8 \times 10^{-4}$
ethyl *p*-aminobenzoate (Benzocaine)	$3{\cdot}6 \times 10^{-5}$
Novocaine	$5{\cdot}8 \times 10^{-8}$
p-hydroxybenzoic acid	—
p-toluic acid	—
benzoic acid	—
benzamide	—
p-aminobenzamide	$1{\cdot}4 \times 10^{-6}$
2-(*p*-aminobenzylamino) pyridine	$0{\cdot}9 \times 10^{-3}$
p-hydroxylaminobenzoic acid*	$5{\cdot}8 \times 10^{-8}$
p-aminophenol	—†
p-aminophenylarsonic acid (arsanilic acid)	—
sulphanilic acid	—‡

— Indicates substance inactive at 10^{-3} *M*.
* It is difficult to be sure that this compound is free from traces of *p*-aminobenzoic acid.
† Inhibits growth down to $3{\cdot}6 \times 10^{-5}$ *M*.
‡ Inhibits growth at 10^{-3} *M*.

recrystallizations the acid gave the M.P. and analytical figures already quoted (p. 84).

p-*aminobenzamide.*—*p*-nitrobenzoic acid was converted to *p*-nitrobenzamide and the latter reduced to *p*-aminobenzamide by the method of Reichenbach and Beilstein (1864). M.P., 180° C.; N, 20·4 per cent. (calc.: 20·5).

p-*hydroxylaminobenzoic acid* was prepared as an aqueous solution according to Goldschmidt and Larsen (1910), and the solid obtained and purified by the method given for the *o*-isomer by Bamberger and Pyman (1909). The substance, which is almost white when freshly prepared, darkens on exposure to light and becomes oxidized in alkaline solution. For testing, the solution (pH 7·8–8) was made up just before sterilizing and used immediately. It was kept in the dark during manipulations.

Other substances used were obtained commercially.

Results.

In general both carboxylic and amino groups in *p*-positions to one another appear to be necessary for anti-sulphanilamide activity (Table IX). Acetylation of the amino group of *p*-aminobenzoic acid leads to a ten-thousandfold decrease in activity, and ethyl esterification of the carboxylic group to a decrease of a thousandfold. These facts were useful in comparing the properties of *p*-aminobenzoic acid with those of the yeast factor. It has already been shown (Table V) that there is a large decrease of activity when the latter is acetylated or ethyl esterified and that the activity is regained on hydrolysis.

Novocaine (diethylaminoethylalcohol ester of *p*-aminobenzoic acid) appears to have the same order of activity as the free acid. The effect, however, was slightly delayed, so it is possible that novocaine is first hydrolysed by the organism.

p-*aminobenzamide*, which is even more closely related to sulphanilamide, is also active, though 100 times less so than *p*-aminobenzoic acid. Its chemical properties are also less in accord with the yeast factor.

p-*nitrobenzoic acid and p-hydroxylaminobenzoic acid.*—It has been reported (Mayer, 1937; Mayer and Oechslin, 1939) that *p*-nitrobenzenesulphonamide and *p*-hydroxylaminobenzenesulphonamide have greater *in vitro* inhibitory action than sulphanilamide itself, and it was suggested that both sulphanilamide and *p*-nitrobenzenesulphonamide are converted to *p*-hydroxylaminobenzenesulphonamide, which was considered to be the true inhibitor. If this is so it was thought possible that the corresponding –COOH analogues (*p*-hydroxylaminobenzoic acid and *p*-nitrobenzoic acid) might be even more active than *p*-aminobenzoic acid in antagonizing sulphanilamide. This did not seem to be the case. *p*-nitrobenzoic acid had only slight anti-sulphanilamide activity; this may be due to slight reduction by the organism to the active amino derivative. Mayer and Oechslin (1939) have also found that *p*-nitrobenzoic acid itself inhibits growth of the streptococcus *in vivo*, though, as in the present experiments, this was not demonstrable *in vitro*.

p-*hydroxylaminobenzoic acid* appeared to have comparable activity to *p*-aminobenzoic acid. It is possible that this compound contained traces of *p*-aminobenzoic acid, but improbable that all the activity can be ascribed to this. The hydroxylamino derivative is liable to oxidation at the pH of the

test, so it is difficult to be certain (*a*) that the concentration remains constant, or (*b*) whether the activity is due to the compound or to its oxidation products.

2-(p-*aminobenzylamino*) *pyridine** is the –CO.NH– analogue of M. & B. 693, and is related to the latter in the same way that *p*-aminobenzamide is related to sulphanilamide. It did not antagonize either sulphanilamide or M. & B. 693.

p-*aminophenylarsonic acid* (*arsanilic acid*) *and* p-*aminophenol* were tried from the point of view of replacing the –COOH of *p*-aminobenzoic acid by another acidic group. It was also thought that these substances might be inhibitory (since replacing –COOH by the acidic $-SO_3H$ or $-SO_2NH_2$ gives rise to an inhibitory substance). Arsanilic acid has been used in the chemotherapeutic treatment of sleeping sickness; it was inactive either as inhibitor or anti-sulphanilamide agent with the streptococcus. *p*-aminophenol, which rapidly oxidizes under the test conditions, has considerable inhibitory action (Table IX), but as this is not reversed by *p*-aminobenzoic acid the inhibition is presumably of a different type.

POSSIBLE IDENTITY OF YEAST FACTOR WITH *p*-AMINOBENZOIC ACID.

All the properties of the yeast extract so far examined correspond with those of *p*-aminobenzoic acid. It was calculated from the relative activities of the two materials that the possible concentration of *p*-aminobenzoic acid in Extracts A and B was $1–2 \times 10^{-3}$ *M*.

Solutions of the acid of this range were therefore used in comparing precipitation by Hg and phosphotungstic acid, norite adsorption, etc., and the conditions used in the yeast experiments were closely followed. The yeast factor corresponded with *p*-aminobenzoic acid in the following properties:

(1) Stability to heat and acid and alkaline treatment.

(2) Presence of weakly acidic group, probably carboxylic (the acid pK of *p*-aminobenzoic acid is 4·9; cf. 4–5 deduced for the yeast factor).

(3) Probable presence of amino group. It will be recalled that purified yeast extracts gave a red colour on diazotizing and coupling with dimethyl-α-napthylamine. This colour was similar in tone to that obtained with *p*-aminobenzoic acid, and it was found that the intensity of the colour was of the same order as that produced by *p*-aminobenzoic acid of concentration equal to that calculated to be present in the extract.

(4) Acetylated and esterified with loss of activity which is regained on hydrolysis.

(5) Precipitated by mercuric acetate, but not by phosphotungstic acid.

(6) Free acid soluble in alcohol, ether and ethyl acetate, but not in petrol ether.

(7) Strongly adsorbed by norite charcoal.

(8) Similar biological activity in all respects.

Although this similarity in many different properties provides strong circumstantial evidence for identity, it must be emphasized that *p*-aminobenzoic acid has not yet been obtained from yeast and that final proof lies in an actual isolation. This is being attempted. The yeast factor may be a different substance, though probably similar in structure and properties.

* I am indebted to Messrs. May and Baker for a sample of this substance.

A POSSIBLE MECHANISM OF SULPHANILAMIDE INHIBITION.

The present investigation was based on a general working hypothesis that anti-bacterial substances act by interfering with some substance essential to the bacterial cell ("essential metabolite," Fildes, 1940). The experiments are also in accord with the suggestion, based on indirect evidence, that the interference is connected with inactivation of bacterial enzymes (Lockwood, 1938; McIntosh and Whitby, 1939), and provide strong evidence that the inactivation is due to competition for an enzyme between the essential metabolite and the inhibitor. A clearer hypothesis of the possible mode of action of sulphanilamide may now be built up and may prove useful as a basis for further work. Throughout the following argument *p*-aminobenzoic acid should be taken to include the probably related, if not identical, naturally occurring materials, such as yeast factor or Stamp factor.

In the first place it is suggested that *p*-aminobenzoic acid is essential for the growth of the organism. It is, however, normally synthesized in sufficient quantity by the present strain of streptococcus (and by *coli*), since it is not necessary to add it to a medium containing only known substances or preparations known to be free from anti-sulphanilamide activity (McIlwain, unpublished). It can also be extracted from the streptococcal cell (Stamp, 1939). On the basis of the experimental work it is next suggested that the enzyme reaction involved in the further utilization of *p*-aminobenzoic acid is subject to competitive inhibition by sulphanilamide, and that this inhibition is due to a structural relationship between sulphanilamide and *p*-aminobenzoic acid (which is the substrate of the enzyme reaction in question). Examples of similar competitive inhibition have already been quoted. It was found that the concentration of *p*-aminobenzoic acid required to overcome this inhibition is 1/5000–1/25,000 of the concentration of sulphanilamide used. The further course of events in a culture may now be considered as follows:

(1) p-*aminobenzoic acid is present preformed in the medium.* Growth occurs. As the anti-sulphanilamide factor appears to be widely distributed in small amount (Table II), this may account in part for the difficulty in getting complete inhibition on more complex media.

(2) p-*aminobenzoic acid is absent from the medium or present in insufficient concentration.* This would be the position under the test conditions used here. There are now two possibilities:

(2*a*) *The organism is unable to synthesize enough* p-*aminobenzoic acid.* Growth is therefore inhibited. This would normally be the case with the streptococcus under the conditions used in the present experiments.

(2*b*) *The organism is able to make sufficient* p-*aminobenzoic acid.* In this case the competitive inhibition is overcome and growth occurs. This is presumed to be the case with organisms that are insensitive to sulphanilamide.

The conditions determining whether (2*a*) or (2*b*) shall take place are delicately balanced, and this may explain why different organisms, and even the same organism under differing growth conditions, exhibit many degrees of sensitivity to sulphanilamide. It is here suggested that such differences in sensitivity are correlated with quantitative differences in ability to synthesize

p-aminobenzoic acid. On meat infusion broth (in which its growth rate approaches optimum) *Bact. coli* is almost indifferent to sulphanilamide, whilst on the ammonium lactate medium (where the growth rate is sub-optimal) inhibition is well marked. Similarly, complete inhibition of streptococcal growth is not obtained (*a*) with rich media on which growth is very rapid, or (*b*) with poorer media if the inoculum is large or consists of young actively dividing cells (Table I). To account for such variability it is suggested that the original inoculum contains sufficient *p*-aminobenzoic acid to reverse the inhibition and permit some (non-visible) growth to take place ; such growth is known to occur in the early stages of inhibition. When this supply of *p*-aminobenzoic acid becomes exhausted by the further enzyme reaction under discussion, subsequent growth will depend on the rate at which more can be synthesized, and this in turn on the number of organisms present and thus on the initial growth rate. Presence of precursors of *p*-aminobenzoic acid in the medium may also influence the rate of synthesis.

Another possible interpretation of the experimental results is that sulphanilamide inhibits the enzyme reaction involved in the *synthesis* (and not, as above, the further utilization) of *p*-aminobenzoic acid, and that it does so this time by virtue of its chemical similarity to the product of the reaction. The balance of evidence is against this view, as in this case it would be expected, on any simple interpretation, that the addition of just sufficient *p*-aminobenzoic acid for the needs of the organism should cause growth ; the amount of *p*-aminobenzoic acid needed should thus be independent of sulphanilamide concentration.

SUMMARY.

(1) Yeast extracts contain a substance which reverses the inhibitory action of sulphanilamide on the growth of haemolytic streptococci.

(2) Examination of the chemical properties of this substance and its behaviour in growth tests suggested that it might be chemically related to sulphanilamide.

(3) *p*-aminobenzoic acid has high activity in antagonizing sulphanilamide inhibition.

(4) There is strong circumstantial evidence that the yeast factor may be *p*-aminobenzoic acid.

(5) On the basis of these results a suggestion is put forward regarding the possible mode of action of sulphanilamide.

The work was carried out at the suggestion of Dr. P. Fildes, to whom I am deeply indebted for advice and criticism throughout. I am grateful to all members of the Department and to Dr. G. M. Richardson for suggestions and to Mr. D. E. Hughes for valuable assistance with some of the chemical work.

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THE INHIBITION OF THE ACTION OF SULPHANILAMIDE IN MICE BY *p*-AMINOBENZOIC ACID.

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MCINTOSH and WHITBY (1939), taking into consideration the lag phase, the inhibition by peptone and the variability of the anti-bacterial action of the sulphanilamide drugs, concluded that these drugs acted by blocking the vital food supply of bacteria probably by the inactivation of a bacterial enzyme, of which a vital food factor is the substrate. The theory that such an enzyme reaction is involved has attracted other workers, and has been largely based on the now well-established fact that the anti-bacterial action of sulphanilamide in bacterial cultures is inhibited by a substance or substances present in peptone and in bacterial extracts (Lockwood, 1938 ; McIntosh and Whitby, 1939 ; Stamp, 1939 ; Fleming, 1940 ; Green, 1940). Woods (1940) now finds that *p*-aminobenzoic acid inhibits the action of sulphanilamide in bacterial cultures. In the present communication it will be shown that *p*-aminobenzoic acid can inhibit the therapeutic action of sulphanilamide in mice infected with streptococci.

METHODS.

The organism used was the Richards strain *Streptococcus haemolyticus*, and the general technique of the experiments followed that of Whitby (1937). The dose of streptococci chosen for inoculation in the therapeutic experiments was the number (500,000) just sufficient to kill all the untreated mice within 24 hours. The drugs were delivered directly into the oesophagus with a metal cannula fitted to a syringe. A 10 per cent. aqueous solution of the sodium salt of *p*-aminobenzoic acid (prepared by Dr. D. D. Woods) was used, so that it could be added to doses of sulphanilamide with no increase in the

bulk of the doses. Sulphanilamide was used throughout as a 10 per cent. suspension in 50 per cent. mucilage.

RESULTS.

In Table I it is shown that the survival rate of mice infected with streptococci and treated with sulphanilamide in doses of 25 mg. can be greatly reduced by the simultaneous administration of *p*-aminobenzoic acid in doses of 2·5, 5·0 and 25 mg. It will be seen in Tables II and III that the toxic dose of *p*-aminobenzoic acid for mice is between 100 and 200 mg., and that in doses of 25 and 30 mg. it has no appreciable effect on the course of the infection. It is also shown in Table IV that the combined toxicity of the two drugs in the conditions of the experiments is, if anything, less than the toxicity of sulphanilamide alone. The results shown in Table I, therefore, cannot be attributed to the toxicity of the drugs or to the influence of the *p*-aminobenzoic acid alone on the course of the infection, but can only be accounted for as due to an inhibition of the antibacterial action of sulphanilamide by *p*-aminobenzoic acid.

TABLE I.—*The Effect of* p-*Aminobenzoic Acid on the Therapeutic Action of Sulphanilamide.*

Experiment.	Doses of drugs in mg.		Number of mice.	Number of mice dying on day:				Survived 7 days.
	Sulphanilamide.	*p*-Aminobenzoic acid.		1.	2.	3.	4.	
1	—	—	6	6	–	–	–	0
	25	—	6	1	1	0	0	4
	25	25	6	6	–	–	–	0
	25	2·5	6	2	3	0	0	1
2	—	—	6	6	–	–	–	0
	25	—	6	0	0	0	0	6
	25	5	6	1	3	0	0	1

All the mice were inoculated intraperitoneally with 500,000 streptococci of the Richards strain from a 6-hour culture in O'Meara's broth diluted to contain the required dose in 0·3 c.c.

The drugs were given by mouth at the time of inoculation, 7 hours later, and then daily.

TABLE II.—*Toxicity of* p-*Aminobenzoic Acid for Mice.*

Experiment.	Total dose of *p*-aminobenzoic acid in mg.	Number of mice.	Number of mice dying on day:				Survived 4 days.
			1.	2.	3.	4.	
1. 2 doses of 50 mg. at 1 hrs.' interval	100	5	0	0	0	0	5
2. 1 dose	100	3	0	0	0	0	3
3. 2 doses of 100 mg. at 1 hrs.' interval	200	3	3	–	–	–	0
4. 3 doses of 100 mg. at 1-hr. intervals	300	3	3	–	–	–	0

TABLE III.—*Effect of* p-*Aminobenzoic Acid on the Lethal Action of Streptococci in Mice.*

Experiment.	Doses in mg. of *p*-amino-benzoic acid.	Number of mice.	Number of mice dying on day:				Survived 7 days.
			1.	2.	3.	4.	
1	—	6	0	4	0	0	2
	30	6	3	2	0	0	1
2	—	5	2	2	0	0	1
	25	5	1	4	–	–	0

All the mice were inoculated intraperitoneally with 50,000 streptococci of the Richards strain from a 6-hour culture in O'Meara's broth diluted to contain the required dose in 0·3 c.c.

The drug was given by mouth at the time of inoculation, 7 hours later, and then daily.

TABLE IV.—*Effect of* p-*Aminobenzoic Acid on the Toxicity of Sulphanilamide.*

Experiment.	Total doses of drugs in mg.		Number of mice.	Number of mice dying on day:				Survived 4 days.
	Sulphanila-mide.	*p*-Amino-benzoic acid.		1.	2.	3.	4.	
1. 1 dose	50	—	5	1	0	3	0	1
	50	10	5	0	0	0	0	5
2. 2 doses at 3 hrs.' interval	100	—	6	6	–	–	–	0
	100	20	6	4	0	0	0	2

DISCUSSION.

The experiments just described show that the therapeutic action of sulphanilamide in mice infected with streptococci can be inhibited by *p*-aminobenzoic acid in the same way as its antistreptococcal action is inhibited in bacterial cultures. The mechanism of its action in vivo would therefore appear to be similar, if not identical to its action in vitro. Woods (1940) has suggested that *p*-aminobenzoic acid or some similar substance is essential for the growth of bacteria and that its utilization can be blocked by sulphanilamide. Many of the discrepancies which have arisen in the course of the work on the sulphanilamide drugs can be explained as due to variations in the amount of the essential substance available in the bacterial cells or in their environment (blood-stream, tissues, etc.). It would also appear probable that the lag phase in the action of sulphanilamide coincides with the slow exhaustion of the essential substance.

SUMMARY.

The therapeutic action of sulphanilamide in mice infected with streptococci can be inhibited by *p*-aminobenzoic acid.

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RAPID METHODS FOR THE DETERMINATION OF UREA IN BLOOD AND URINE.

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IN spite of the many ingenious and laborious investigations that have been carried out in an endeavour to elucidate and simplify the blood urea method the procedure has always retained a somewhat formidable and time-consuming technique. The object of the present paper is to provide quite a simple method which can be carried out by any operator having only an elementary knowledge of chemical methods and manipulation. It also possesses the great advantage that it is possible to obtain an accurate estimation of blood urea in 20 minutes, without the use of elaborate apparatus at present employed by the methods now in vogue.

The process can be performed in two operations, which may be summarized as follows :

1. The blood protein is removed by precipitation with alcohol or acetone, and after filtration or centrifugalization, the protein-free extract is diluted with water and adjusted to a very faintly acid reaction with N/100 sulphuric acid, using a special methyl red-methylene blue mixture as indicator.

2. A specially powerful neutral extract of "Urease-Dunning" is added to the blood filtrate mixture, which, after thorough mixing in a well-stoppered tube, is heated in a water bath at 50 to 55° C. for 10 to 15 minutes. The urea present is quantitatively converted into ammonium carbonate, which in turn is titrated with N/100 sulphuric acid to the original faintly acid starting-point of the special indicator.

The amount of N/100 acid added is directly proportional to the urea originally present, which is superior to the back titration methods.

The principle adopted here for the estimation of urea in blood can be applied directly to urine by a simple 1 in 50 dilution, of which 5 c.c. is taken and adjusted to the faintly acid side of the special indicator with N/100 sulphuric acid, after which it is acted upon by the neutral urease extract. The ammonium carbonate formed by immersion in the water bath at 50 to 55° C. for 5 minutes is titrated back to the original slightly acid starting-point of the reaction with N/20 sulphuric acid. The determination can therefore be quantitatively carried out in less than 10 minutes, being more rapid and simple in technique than the method described by Cole (1937).

REAGENTS AND APPARATUS.

The reagents and apparatus required for the method are :

1. Centrifuge tubes graduated at 5 c.c., and thick-walled test tubes of approximately 6 × 5/8 in.
2. Pipettes of 0·2 c.c., 1 c.c., 2·5 c.c. and 3·5 c.c. capacity.
3. Two 5 c.c. micro-burettes graduated to 0·05 c.c.
4. N/20 and N/100 sulphuric acid.
5. Approximately N/50 sodium hydroxide.
6. No. 1 Whatman filter papers of 5·5 cm. diameter.
7. Absolute alcohol and redistilled or " Analar " acetone.
8. Special mixed indicator :

Stock solution A : 0·02 per cent. methyl red in 60 per cent. (by volume) absolute alcohol. The solid methyl red should be ground up with absolute alcohol in a small mortar till completely dissolved, and transferred to the 100 c.c. flask by repeated washings with absolute alcohol in the usual manner. 40 c.c. of water is added and the volume brought to 100 c.c. with absolute alcohol.

Stock solution B : 0·10 per cent. aqueous methylene blue.

The indicator is prepared by adding 1 c.c. of the methylene blue (solution B) to 15 c.c. of methyl red (solution A). The mixture, if green in colour, is brought to a very faintly acid reaction by adding N/10 hydrochloric acid drop by drop with constant shaking until the solution just turns reddish-purple. This solution keeps indefinitely if stored in an amber ground glass stoppered bottle. It is reddish-purple in acid solution, and bright green in alkaline solutions.

9. Neutral " Urease-Dunning " enzyme suspension.

Four 25 mg. tablets of " Urease-Dunning "* are thoroughly ground up in a small mortar with 4·5 c.c. of N/100 sulphuric acid. When an even emulsion has been obtained 0·2 c.c. of the mixed indicator is added. The extract, if correctly prepared, has a greyish-buff coloration (the neutral point of the indicator) and keeps in the refrigerator one month.

* Obtained from Hynson, Westcott & Dunning, of Baltimore, U.S.A., or from Baird & Tatlock, Hatton Garden, London.

METHODS.

Estimation of Urea in Blood.

Into one of the 5 c.c. graduated centrifuge tubes, containing 3–4 c.c. of absolute alcohol or acetone, pipette 1 c.c. of serum, oxalated whole blood, or plasma. As it is impossible to remove the last traces of blood from the pipette by sucking up and blowing out with alcohol in the usual manner (due to the pipette becoming clogged with precipitated blood protein) the pipette must be allowed to drain thoroughly for about 1 minute, and the last traces of blood removed by expelling with air. By this procedure not more than 0·01 c.c. of blood is retained in the pipette. The centrifuge tube is now securely stoppered and vigorously shaken, after which the volume is readjusted to 5 c.c. by the further addition of a few drops of absolute alcohol or acetone, as the case may be, to counteract the reduction in volume due to the formation of alcoholic hydrates. Mix again by shaking well and centrifuge at high speed for 2–3 minutes, or filter through a No. 1, 5·5 cm. paper. After filtration the precipitated blood protein contained in the filter paper should be disturbed by very gentle stirring with a small glass rod, otherwise the 2·5 c.c. of filtrate may not always be obtained from bloods rich in meta-protein. Measure 2·5 c.c. of supernatant fluid or filtrate into one of the thick-walled test-tubes and add 3·5 c.c. of water. The alcoholic extract becomes slightly opalescent due to the partial precipitation of blood fat by the lowered alcoholic concentration. 0·3 c.c. of special indicator is added and the alkaline reaction (green) brought over to the acid side (reddish-purple) by the addition of N/20 sulphuric acid, with thorough mixing after the addition of each drop. (The alcoholic extract varies in alkalinity according to the blood bicarbonate content.) N/50 sodium hydroxide is carefully added until the reaction is just alkaline again, and the reaction finally brought over to the very faintly acid point of the indicator by the addition of N/100 sulphuric acid, drop by drop, with constant shaking. By this readjustment of the pH, final titration with relatively strong acid is avoided, enabling the starting-point of the reaction to be controlled to within one drop of N/100 sulphuric acid, which in terms of blood urea is approximately 1 mg. per 100 c.c. Finally, 0·35 c.c. of well shaken neutral urease suspension is added, and after thorough mixing, the tube is securely stoppered and heated in the water bath at 50 to 55° C. for 10–15 minutes. Remove the tube from the bath and titrate with N/100 sulphuric acid until slightly acid (the original reddish-purple starting-point of the reaction). The titration value in c.c. multiplied by 60 equals mg. of urea per 100 c.c. of blood.

With the above method it is necessary to emphasize the importance of using only samples of serum, oxalated whole blood or plasma for analysis.

The addition of sodium fluoride as preservative and anticoagulant renders the analytical process useless, because of its inhibitory action on the enzyme. For other body fluids, such as C.S.F., transudates, exudates, etc., proceed exactly as for blood urea.

Table I shows a comparison between the results obtained by the process

just recorded and the standard classical aeration method of Patterson (1925) as used in the Charing Cross Hospital and Dorset County Laboratories.

TABLE I.—*Mg. of Urea per* 100 *c.c. Blood.*

N.	O.	N.	O.	N.	O.	N.	O.	N.	O.	N.	O.
10	9	24	24	32	34	51	52	168	176	311	327
12	15	25	24	33	35	60	58	182	181	363	344
13	15	27	26	36	38	74	72	201	207	438	432
18	19	29	27	42	43	99	99	246	249	474	472
20	22	30	30	48	48	151	150	288	297	528	530

N = New. O = Old method.

Estimation of Urea in Urine.

For the estimation of urea in urine a 1 in 50 dilution of the specimen is made with distilled water (with small quantities of urine 0·2 c.c. can be measured with a blood pipette into 9·8 c.c. of water), and after mixing well, 5 c.c. is pipetted into a thick-walled test tube and 0·3 c.c. of indicator added. The faint greenish coloration obtained with the majority of urines is due to the urine being alkaline to the special indicator. Adjustment is made to the faintly acid side of the indicator by the addition of N/100 sulphuric acid drop by drop. 0·5 c.c. of well shaken neutral urease suspension is now added, the tube is securely stoppered, and the mixture (which turns green almost immediately) is heated in the water bath at 50° to 55° C. for 5 minutes. The ammonium carbonate so formed is back titrated with N/20 sulphuric acid till just reddish-purple, i.e. the original starting-point of the reaction. The titration value in c.c. multiplied by 1·5 equals grammes of urea per 100 c.c. of urine.

Table II epitomizes the results of the urinary process compared against the hypobromide method of Garrod as modified by MacLean (1921).

TABLE II.—*Showing Urine Urea in gm. per* 100 *c.c.*

New method.	Old method.
0·37	0·37
0·58	0·59
0·93	0·91
1·20	1·30
2·85	2·85
3·27	3·25
4·87	4·87
5·81	5·79

The satisfactory agreement of the blood and urine results with the values obtained by the classical procedure establishes confidence in the new methods as a means of determining urea in body fluids.

SUMMARY.

A simple quantitative method is described for the routine determination of urea in blood, the fundamental principle being based on the removal of the blood protein with alcohol or acetone, and the treatment of the alcoholic extract with a neutralized concentrated suspension of Urease-Dunning at 50° C. for 10–15 minutes. The ammonium carbonate so formed is titrated with N/100 sulphuric acid in the presence of a special mixed indicator.

The process is both rapid and delicate, enabling an estimation to be made in 20 minutes, with a range of from 5–500 mg. per 100 c.c., the experimental error being of the order of $\pm$ 2 mg.

The method can be applied directly to urine, using a 1 in 50 dilution. For other body fluids the blood urea method is followed in detail.

This new approach greatly simplifies the urease principle of blood urea determinations, thus avoiding time-consuming methods, such as distillation, aeration, or special gasometric methods, nearly all of which employ elaborate apparatus.

I wish to express my thanks to Dr. J. Patterson, Biochemist to Charing Cross Hospital, for his interest and encouragement during part of this work, and to Dr. T. W. Stallybrass, County Medical Officer of Health for Dorset, and Dr. T. V. Cooper, Pathologist to the County Laboratory, Dorchester, for permission to conclude the investigation at the County Laboratory.

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QUANTITATIVE INVESTIGATION OF CHANGES IN ANTIGENIC PROPERTIES IN THE COURSE OF IODINATION OF HORSE-SERUM GLOBULIN.

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DURING iodination of proteins the new iodine-specificity appears at an early stage of iodination, that is, when the amount of organically bound iodine reaches about 1 per cent. of the weight of the protein (Haurowitz and Appel, 1939). The species specificity is nevertheless preserved: the ability to react with, and to produce corresponding antisera to native proteins is still marked. Further introduction of iodine eventually leads to complete loss of species specificity (Wormall, 1930).

In the following work an attempt has been made to correlate the degree of iodination of horse-serum globulin with disappearance of species specificity. Quantitative investigation also has been made of the precipitin reaction between partly iodized horse-serum globulin and the corresponding antisera to native globulin.

METHODS.

Iodination of globulin was carried out in alkaline and in neutral solution. In alkaline solution the procedure was similar to that described by Wormall (1930), but in some cases proportionately more iodine solution was added than he used.

To 20 ml. of 3·4 per cent. horse-serum globulin 10 ml. NH_4OH were added, followed by $N/10$ I–KI solution in portions of 4 ml. at intervals of about ½ hour. Afterwards acetic acid was added in amount sufficient to cause maximal precipitation; the precipitate was centrifuged off and dissolved in water by adding Na_2CO_3 to pH not greater than 8·5. Precipitation and dissolution were repeated several times until the test for free iodine with starch solution was entirely negative.

Iodination in neutral solution was performed in a similar way, except that instead of NH_4OH $M/4$ phosphate buffer solution (pH 7·3) was used and $N/10$ iodine solution was added in portions of 1 ml.

Total iodine estimations were carried out by the method described by Herriot (1937). Throughout this paper iodine is recorded as percentages of the weight of protein.

The *extent of substitution of tyrosine*, which is correlated with fall of Folin colour value, was measured colorimetrically with Folin's phenol reagent.

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The amount of iodoprotein, which gives approximately the colour produced by 0·15 mg. of tyrosine, was diluted with water to 19 ml.; 3 ml. of 1·28 *N* sodium hydroxide were added followed by 3 ml. of 1 : 3 dilution of Folin's phenol reagent. The solution was allowed to stand 5 minutes for the colour to develop, after which it was compared in a colorimeter against native globulin solution treated in a like manner.

The amounts of precipitate were measured by the method described by Marrack and Duff (1938):

Different concentrations of antigen solution (adjusted to a constant volume) were mixed with a constant volume of an antiserum at 0° C.; the mixtures were kept overnight in the refrigerator; the precipitates were centrifuged off, washed with cold 1 per cent. NaCl solution, and the total nitrogen was estimated by the Kjeldahl method.

(Throughout this paper all the amounts of iodo-proteins, as well as precipitates containing the iodine, are given without taking into account the iodine weight.)

Rabbits were immunized by intravenous injections of 4 ml. of 2 per cent. iodo-protein twice a week for four weeks, and with normal globulin by injections of 4 ml. of 2 per cent. globulin solution twice a week for three weeks.

ESTIMATION OF THE DEGREE OF IODINATION AT WHICH SPECIES SPECIFICITY DISAPPEARS.

Reaction with Anti-sera to Native Globulin.

Iodination in neutral solution never resulted in fall in Folin colour value more than to about 70 per cent. of the original value, and the precipitin reaction with antisera to native globulin was always strongly positive. Iodination in alkaline solution caused a complete loss of the ability to react with those sera, when the fall in Folin value was to about 30 per cent. (that was the maximal fall after iodination in alkaline solution). When the fall in Folin value was to 40 per cent. of the original value, the precipitin reaction with antisera to native globulin was still strongly positive.

Reaction of Different Antisera to Iodo-globulin.

In Table I are given the results of qualitative precipitin tests of three antisera produced by immunization of rabbits with iodized horse-serum globulin (*A*), the ability of which to react with antisera to native globulin was preserved, and of three antisera produced by iodized globulin (*B*), the ability of which to react with antisera to native globulin was lost.

All the sera reacted strongly with both kinds of iodized horse-serum globulin, but more strongly with horse globulin (*A*) iodized in neutral solution than with that iodized in alkaline solution (*B*). Not all the sera reacted with iodized rabbit serum protein or with iodized crystalline egg albumin, and those which did react showed a weaker reaction than that given with iodized horse-serum globulin.

TABLE I.

Antigen.	Antiserum produced by Iodized horse-serum globulin. A. No. of serum. 1.	2.	3.	Iodized horse-serum globulin. B. No. of serum. 2.	5.	6.
Native horse-serum globulin	++	++	±	—	—	±
Iodized horse-serum globulin *A*	+++	+++	+	+++	+++	++
,, ,, ,, *B*	+	+++	+	+++	+	++
,, rabbit serum protein	+	++	—	±	—	—
,, crystalline egg albumin	±	++	—	±	±	—
Normal crystalline egg albumin	—	—	—	—	—	—

Number of + signs indicates the degree of precipitation.

A. Horse-serum globulin iodized in neutral solution. Iodine content 5·2 per cent. of protein weight: Folin colour value 70 per cent. of the original value. *Reaction with anti-serum to native globulin strongly positive.*

B. Horse-serum globulin iodized in alkaline solution. Iodine content 11 per cent. of protein weight: Folin colour value 30 per cent. of the original value. *Reaction with antiserum to native globulin entirely negative.*

All the antisera, produced by iodized horse globulin (*A*) which had not lost the ability to react with antisera to native globulin, reacted with normal horse-serum globulin. Two of three antisera produced by iodized horse globulin (*B*) which had lost the ability to react with antisera to native globulin gave no reaction with normal horse globulin (sera 4 and 5) except one which gave a very weak reaction (serum 6).

THE AMOUNTS OF PRECIPITATE FORMED BY IODIZED GLOBULIN WITH ANTISERUM TO NORMAL GLOBULIN.

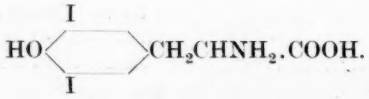
The results of estimations of the amount of precipitate formed by an antiserum to native globulin (534.24.2.39) with an iodized horse-serum globulin, which had not lost the ability to react with antisera to native globulin, and with native globulin are given in Fig. 1.

In comparison with the amount of precipitate formed with native globulin the iodized globulin in the excess antibody zone forms less precipitate, but in the excess antigen zone it forms much more precipitate. Thus the possible maximum amount of precipitate obtainable from a given volume of an antiserum is greater with iodized than with native globulin.

The estimation of optimal proportions (Dean and Webb, 1926) showed no difference between native and iodized globulin, being in both cases about 1·5 mg. of protein to 1 ml. of antiserum (534.24.2.39).

DISCUSSION.

The isolation of 3–5-di-iodotyrosine from artificially iodized proteins by Oswald (1911) shows that iodine actually substitutes the benzene ring of tyrosine in iodized proteins:



The inhibition tests not only with di-iodotyrosine, but also with other aromatic compounds containing two atoms of iodine in the benzene ring in *ortho* position to the OH-group (Snapper and Grunbaum, 1936), speak in favour of the conception that the appearance of the new iodine-specificity of iodo-proteins is actually due to substitution of tyrosine.

According to Haurowitz and Appel (1939) this new specificity appears as soon as the iodine content reaches about 1 per cent. of the protein weight, and this implies that substitution of a small fraction of total tyrosine is sufficient to confer the new specificity.

As we have shown, the species specificity disappears when the Folin colour value falls to about 30 per cent. of the original value. If we assume according

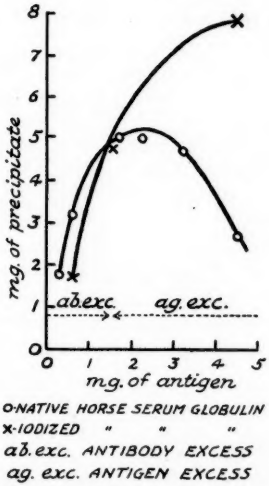


FIG. 1.—*The amounts of precipitate formed by an antiserum to native globulin* (534.24.2.39) *with native and with iodized horse-serum globulin.* Iodized horse-serum globulin corresponds to *A* (Table I). Antigen and antibody excess means positive precipitin test of supernatant fluid with antiserum or antigen respectively.

to Holiday (1936) that the amount of tyrosine in whole horse-serum globulin is about three times greater than the amount of tryptophan, and if we take into account that tryptophan gives with Folin's phenol reagent 0·887 times as intense colour as tyrosine (Folin and Ciocalteu, 1927), then it follows that fall in Folin value to about 30 per cent. may correspond to substitution of all tyrosine, the remaining 30 per cent. being due exclusively to tryptophan.

Now the question arises, whether the disappearance of species specificity is exclusively due to substitution of tyrosine by iodine, or whether some other changes are also playing a causative role.

Besides substitution of tyrosine it is possible to consider the possibility of other substitutions, which could be responsible for the loss of species specificity (according to Bauer and Strauss (1929) histidine can be substituted). However, the following calculation speaks against this possibility :

If we assume according to Holiday (1936) that tyrosine content of whole horse-serum globulin is about 7 per cent., and if we take the molecular weight of tyrosine *in* protein molecule as 163 (= 181 – 18 (H_2O)), then the amount of iodine necessary to substitute all the tyrosine would be : $7 \times 254/163 = 10{\cdot}9$, i.e. about 11 per cent. of protein weight. In our case, the iodized globulin (*B*) (Table I) which lost its species specificity showed a Folin colour value 30 per cent. of the original value, which can be interpreted as corresponding to substitution of all tyrosine. The iodine content was 11 per cent. of protein weight, so that nothing remains to account for the possibility of other substitutions.

This does not exclude the possibility of changes of another kind which could be responsible for loss of species specificity, but as we are at present not aware of them, we can accept as a working hypothesis that substitution of tyrosine alone leads to loss of species specificity.

Immunization with iodo-proteins, according to Haurowitz and Appel (1939), gives rise to production of two kinds of antibodies : (1) those reacting with homologous iodo-proteins only and (2) those reacting with all iodo-proteins. The results shown in Table I seem to confirm this. All the antisera contain the first kind of antibodies (stronger antigenic stimulus) and react with iodized horse-serum globulin, but only some of them contain the second kind of antibodies and react with iodized proteins of other species (including iodized rabbit serum protein).

The antisera produced by iodized horse-serum globulin (*A*), which retained its ability to react with antisera to native globulin (antisera 1, 2 and 3, Table I), also contain a third kind of antibody, namely anti-species antibodies, which react with native horse globulin.

Both the ability to react with, and the ability to produce anti-species antibodies disappear almost simultaneously during iodination of horse-serum globulin, when presumably all tyrosine is substituted. It may be that the ability to produce anti-species antibodies is preserved during iodination somewhat longer, since it may happen that iodo-globulin, which has completely lost the ability to react with antisera to native globulin, gives rise to production of anti-species antibodies though in negligible amount (antiserum 6, Table I).

In the course of other kinds of treatment of proteins, it may occur that, either the ability to produce anti-species antibodies is destroyed while the ability to react with them is preserved (as it is in the case of treatment with phenyl*iso*cyanate (Hopkins and Wormall, 1933; Kleczkowski, 1940)), or *vice versa* (as it is in the case of certain azo-proteins (Heidelberger and Kendall, 1934)).

Whenever iodized horse-serum globulin retains its ability to react with antisera to native globulin it forms in the excess antigen zone even more precipitate than the normal globulin does. The same phenomenon had been noticed when horse-serum globulin was treated with phenyl*iso*cyanate or formaldehyde, in spite of a manifestation of somewhat reduced affinity towards the antibody (Kleczkowski, 1940). Crystalline egg albumin treated with phenyl*iso*cyanate or formaldehyde did not show this phenomenon. It has already been suggested (Kleczkowski, 1940) that chemical treatment might have changed the physical properties of globulin molecules, and in this way might

reduce or even abolish their ability to form soluble antigen-antibody compounds which are normally formed by untreated globulin molecules especially in the excess antigen zone ; that would account for formation of greater amounts of precipitate in the excess antigen zone.

The phenomenon described above indicates that the maximal amount of precipitate that can be obtained from a given volume of an antiserum cannot always be regarded as a relative measure of the strength of affinity between antigen and antibody.

SUMMARY.

1. In the course of iodination of horse-serum globulin the ability to react with and the ability to produce antibodies to native globulin disappear almost simultaneously when the Folin colour value falls to about 30 per cent. of the original value, i.e. presumably when all the tyrosine is substituted.

2. When the Folin colour value falls only to 40 per cent. or less the iodized globulin reacts strongly with antisera to native globulin, and in the excess antigen zone forms even more precipitate than the native globulin does. This is presumably due to changes in some physical properties of the globulin molecules induced by the treatment.

My thanks are due to Prof. J. R. Marrack for his guidance whilst this work was carried out.

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THE EFFECT OF VACCINATION OF GUINEA-PIGS WITH THE VOLE ACID-FAST BACILLUS ON SUBSEQUENT TUBERCULOUS INFECTION.

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MANY attempts have been made in the past to produce immunity in animals to experimental tuberculous infection. Live and killed vaccines of tubercle bacilli and other *Mycobacteria* have been used, but, with the exception of cattle inoculated with living human type bacilli, no striking degree of immunity has been reported. It is not possible in this short paper to review the extensive literature on the subject.

The object of this paper is to report the results of some experiments we made to determine whether any immunity to experimental infection with virulent human and bovine tubercle bacilli is conferred on guinea-pigs by vaccination with sub-lethal doses of the vole acid-fast bacillus. The disease in wild voles, which closely resembles tuberculosis, has already been briefly described (Wells, 1937). We hope to show in a subsequent paper that the *Mycobacterium* concerned is an undescribed type of tubercle bacillus. We realize that the results reported here are inconclusive and statistically insignificant. We feel, however, that they are sufficiently striking to warrant a preliminary report, especially as the war has seriously interfered with the continuation of our experiments.

EXPERIMENTAL.

It has been found that the vole acid-fast bacillus is only slightly virulent for guinea-pigs. A subcutaneous injection of 5 mg. or more may produce progressive disease and death ; a smaller inoculum produces a local abscess with caseation and sometimes ulceration, but the lesion is regressive. The inoculum in all cases was a suspension made from cultures on Dorset's egg medium.

Experiment 1.

Three guinea-pigs were inoculated subcutaneously in the left groin with caseous material from naturally infected voles. These animals developed a

* Working with a grant from the Medical Research Council.

local lesion which ulcerated, but there was no sign of generalized disease. Nine months later an injection of 0·000,001 mg. of a one month culture of bovine tubercle bacilli of low virulence was given subcutaneously in the right groin. At the same time three normal guinea-pigs received a similar injection. The result of this experiment is shown in Table I.

TABLE I.

	Preliminary injection.	Survival after injection.	Lesions at post-mortem.
Guinea-pig 1	Vole acid-fast bacillus	Killed 6 months	Small local caseous lesion only.
,, 2	Ditto	Ditto	Ditto.
,, 3	,,	,,	Local caseous lesion; 2 small tubercles in lungs.
,, 4	None	,,	Extensive generalized tuberculosis, involving lungs, liver, spleen and lymph glands.
,, 5	,,	,,	
,, 6	,,	Died 3 weeks	Small local lesion.

The cause of death in Guinea-pig 6, one of the controls, was not found. It was certainly not tuberculosis. The difference in the degree of infection between those animals which had received a preliminary injection of the vole acid-fast bacillus and the controls was very marked. In only one of the former was there any macroscopical tuberculosis, apart from the lesion at the site of the injection. The two controls showed extensive generalized tuberculosis.

Experiment 2.

In this experiment 15 guinea-pigs were used. They were divided into three groups of 5 each. Group 1 were given an injection of 1 mg. of a

TABLE II.

	Number of guinea-pigs in group.	Immunizing injection.	Lesions at post-mortem 3 months after injection of bovine tubercle bacilli.
Group 1	All 5	1 mg. of culture of vole acid-fast bacillus G202	Small local abscess only.
,, 2	1	0·1 mg. of culture of vole acid-fast bacillus G202	Local abscess only.
	2		Ditto.
	3		Local abscess, enlarged but not caseous iliac and portal lymph glands.
	4		Ditto.
	5		Died after 2 months. Large local abscess, caseous inguinal and iliac lymph glands, tubercles in liver and spleen.
Group 3 (controls)	All 5	None	All died before 3 months with extensive generalized tuberculosis.

two-month culture of vole acid-fast bacillus in the left groin. Group 2 were given 0·1 mg. of the same culture of vole acid-fast bacillus in the left groin. Group 3 were controls. Three months later all 15 animals were given 0·000,01 mg. of a one-month culture of recently isolated human tubercle bacilli in the right groin. Table II summarizes the results of this experiment.

The protection afforded by the preliminary vaccination with the vole acid-fast bacillus was again very marked. The five guinea-pigs in Group 1 showed no macroscopical signs of disease in the lymph glands or organs, whereas all the controls had died with extensive generalized tuberculosis. It would seem that the preliminary injection of 1 mg. of culture of vole acid-fast bacillus gives a greater degree of protection than does an injection of 0·1 mg.

Experiment 3.

In this experiment we wanted to compare the degree of protection conferred by the preliminary vaccination of guinea-pigs with two strains of vole acid-fast bacillus and with B.C.G. to subsequent infection with human and bovine tubercle bacilli. Four groups of animals were used. Group 1 consisted of 26 animals, each of which received four weekly injections of a two-month culture of a strain of vole acid-fast bacillus—D 15—subcutaneously in the right and left groins alternately. Each animal received a total of 2 mg. of culture. Group 2 consisted of 26 animals each of which received exactly similar injections of another strain of vole acid-fast bacillus—LV 565. Group 3 consisted of 30 animals each of which received four weekly doses of 7·5 mg. of a one-month culture of B.C.G. subcutaneously in the left and right groins alternately. Each animal received a total of 30 mg. of B.C.G. This dose of B.C.G. was thought to be the optimal dose for giving protection.

Unfortunately intercurrent disease killed a large number of these guinea-pigs, so that after five months Group 1 was reduced to 9 animals, Group 2 to 10 and Group 3 to 8. The disease which killed so many animals was epidemic; its cause was not definitely determined. Except for one animal, from which *Pasteurella septica* was isolated, cultures remained sterile. The experiment was, therefore, to a great extent invalidated.

Five months after vaccination Group 1 received 0·000,001 mg. of a recently isolated one month culture of human tubercle bacilli subcutaneously into the left axilla; Group 2 received 0·000,001 mg. of a recently isolated one-month culture of bovine tubercle bacilli subcutaneously into the left axilla; Group 3 was divided into two lots of 4 animals each; one lot received a dose of human tubercle bacilli similar to Group 1; the other lot received bovine tubercle bacilli similar to Group 2; Group 4 consisted of 16 unvaccinated animals, 8 of which received human tubercle bacilli and 8 bovine tubercle bacilli. All the animals in these four groups, with the exception of two each in Groups 1 and 2, were killed after 11 weeks. It was originally intended to leave all the animals for 18 months, or as long as they survived, to determine the survival time in each group. This procedure was impossible owing to the war interfering with our arrangements. The results are summarized in Table III.

Two of the four guinea-pigs in Groups 1 and 2 which were not killed at

Table III.

	Number of animal in group.	Infecting dose.	Lesions at post-mortem 11 weeks after infecting dose.
Group 1.			
2 mg. vole acid-fast bacillus, D15	1	0·000,001 mg. T.B. human	No lesions found.
	2		Small local abscess only.
	3		Ditto.
	4		,,
	5		,,
	6		Small local abscess, 2 tiny tubercles in lungs.
	7		Local abscess, small tubercles in liver, spleen and lungs.
Group 2.			
2 mg. vole acid-fast bacillus, LV 565	1	0·000,001 mg. T.B. bovine	No lesions found.
	2		Small local abscess only.
	3		Ditto.
	4		,,
	5		,,
	6		,,
	7		Small local abscess, 2 tiny tubercles in lungs.
	8		Local abscess, small tubercles in liver, spleen and lungs.
Group 3.			
30 mg. B.C.G.	1	0·000,001 mg. T.B. human	Generalized tuberculosis; tubercles in lungs, liver, spleen and lymph glands.
	2		
	3		
	4		
	5	0·000,001 mg. T.B. bovine	
	6		
	7		
	8		
Group 4.			
Unvaccinated	1	0·000,001 mg. T.B. human	Extensive generalized tuberculosis.
	2		
	3		
	4		
	5		
	6		
	7		
	8		
	9	0·000,001 mg. T.B. bovine	
	10		
	11		
	12		
	13		
	14		
	15		
	16		

11 weeks died 16 weeks after infection with tubercle bacilli. One was in Group 1 and the other in Group 2. The only macroscopic sign of tuberculous infection found was a small caseous lesion at the site of inoculation in the left axilla of each animal. The remaining two animals are alive, 20 weeks after infection.

The degree of infection present in the four groups 11 weeks after infection with human or bovine tubercle bacilli showed very marked difference. Eleven of the animals in Groups 1 and 2 which had been vaccinated with vole acid-fast bacilli showed either no macroscopic lesion or a small local abscess, and the remaining four showed very early signs of generalized disease. The animals in Group 3 which had been vaccinated with B.C.G. all showed generalized disease, though less advanced than the controls. The post-mortem findings were, however, very different from the animals in Groups 1 and 2.

After infection with tubercle bacilli all the animals were examined weekly. Palpation of the axillae showed that increase in the size of the glands occurred earlier in the controls than in the vaccinated animals. Thus four weeks after infection the glands in the left axilla could be palpated in 15 out of 16 controls, but in only 2 out of 15 animals vaccinated with the vole acid-fast bacillus, and in 5 out of 8 animals vaccinated with B.C.G. Six weeks after infection all of the controls had enlarged glands in the left axilla, but only 7 out of 15 animals vaccinated with vole acid-fast bacillus had tiny palpable glands, and 7 out of 8 animals vaccinated with B.C.G. Ten weeks after infection all the controls and B.C.G. vaccinated animals had enlarged glands, but only in 10 out of 15 animals vaccinated with vole acid-fast bacillus were glands palpable.

Caseous matter at the site of vaccination with vole acid-fast bacillus was present at death in about two-thirds of the guinea-pigs. The presence or absence of this caseous matter did not seem to be related to the degree of immunity.

All the animals were tested with either " Old Tuberculin " or " Purified Protein Derivative " at the beginning and on the third, fourth, eighth and eleventh week after infection with tubercle bacilli. Table IV shows the highest dilution of tuberculin to which the animals reacted. A positive reaction was arbitrarily set as an area of erythema and induration, with or without central blanching or necrosis, of at least 14 mm. in the longest diameter. The readings were taken after 48 hours.

It is seen that there were differences in the development of skin sensitivity between the controls and the vaccinated animals. These differences were in the speed of development and in the degree of sensitivity reached by the 11th week. The control animals showed more positive reactions to the 1 : 100 dilution at the 3rd and 4th weeks than did either of the vaccinated groups, and at the 11th week, 15 out of 16 controls reacted positively to the 1 : 1000 or the 1 : 10,000 dilutions. None of the animals vaccinated with the vole acid-fast bacillus and only 3 out of 8 of the animals vaccinated with B.C.G. became positive to 1 : 1000 tuberculin. These results are similar to those of Schwabacher and Wilson (1938), who found in guinea-pigs vaccinated with B.C.G. and infected with virulent mammalian tubercle bacilli that the tuberculin sensitivity took longer to develop and did not reach so high a level as in the control animals.

TABLE IV.—*Highest Dilution of Tuberculin to which Animals Reacted.*

	Vaccinated with the vole A.F.B. Number reacting to—				Vaccinated with B.C.G. Number reacting to—				Controls. Number reacting to—			
	1:20.	1:100.	1:1000.	1:10,000.	1:20.	1:100.	1:1000.	1:10,000.	1:20.	1:100.	1:1000.	1:10,000.
At time of infection with tubercle bacilli	15	2	0	Not tested.	8	1	0	Not tested.	0	Not tested.		
After 3 weeks	15	6	0	Not tested.	8	6	0	Not tested.	16	8	0	0
After 4 weeks	15	6	0	Not tested.	8	7	0	Not tested.	16	13	0	0
After 8 weeks	15	8	0	Not tested.	8	8	2	0	16	16	14	0
After 11 weeks	15	11	0	0	8	8	3	0	16	16	15	2

SUMMARY.

Vaccination of guinea-pigs with the vole acid-fast bacillus prior to infection with virulent mammalian tubercle bacilli gives a degree of protection which apparently is far greater than has been recorded by other means.

We acknowledge with gratitude the " Purified Protein Derivative " supplied by the Wellcome Physiological Laboratories.

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THE CULTIVATION OF LYMPHOCYTIC CHORIOMENINGITIS IN TISSUE CULTURE.

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SINCE 1928, when Maitland and Maitland showed that vaccinia could be grown readily in a medium containing minced rabbit kidney suspended in chicken serum diluted with Tyrode's solution, many viruses have been cultivated by this method and its various modifications (Li and Rivers, 1930). This technique has been used to provide large supplies of virus for critical studies and immunization purposes, more especially in the case of vaccinia and yellow fever.

In 1936 Bengston and Wooley described the cultivation of the virus of lymphocytic choriomeningitis on the chorio-allantoic membrane of the developing chick embryo. They carried the virus through eight passages and found it in approximately the same amount in the membrane and brain of the embryo. It was also present in the liver and the amniotic fluid. Casals-Ariet and Webster (1940) have recently described the appearance of the virus in their tissue cultures of rabies virus, the contaminant having been introduced either in the serum of monkeys or in the brains of mice with a latent infection of

lymphocytic choriomeningitis. Findlay (1936) suggested the possibility of contamination of tissue cultures of mouse brain with this virus and, because of this danger, changed over from mouse embryo to chicken embryo tissue for cultivation of the yellow fever virus for vaccine production.

The original English strain of lymphocytic choriomeningitis was isolated in 1935 (Findlay, Alcock and Stern, 1936). Cultivation of this virus in tissue cultures in flasks was commenced on January 14, 1936, and carried for 270 subcultures until interrupted in September, 1939. A second strain was carried for 38 subcultures till it was also interrupted in September, 1939. A third strain started in October, 1939, has been subcultured 35 times to date.

METHOD.

The original medium consisted of 4·5 c.c. serum Tyrode's solution (1 part normal inactivated human serum, 9 parts Tyrode's solution) and approximately 0·1 gm. of minced whole 9 or 10-day-old chick embryo placed in 50 c.c. Erlenmeyer flasks with ordinary cork stoppers covered with tinfoil. To this was added 0·5 c.c. of the supernatant fluid of a lightly spun (1000 r.p.m. × 10 minutes) 10 per cent. suspension of infected mouse brain in normal physiological saline solution ; the flasks were incubated at 37° C.

Subcultures were carried out every three to four days using 1·0 c.c. supernatant fluid ; samples of the supernatant fluid were tested for sterility and inoculated intracerebrally into mice on each subculture and from time to time into monkeys and guinea-pigs.

The tissue in the medium was chick embryo for the first 97 subcultures. At this point a change was made, chick embryo brain alone being used for 18 subcultures. Then at the 116th subculture a change was again made and only the tissues remaining after the brain and spinal cord had been removed were used. This latter medium was maintained till the cultivation was stopped, and also throughout the cultivation of the other two strains.

EXPERIMENTAL RESULTS.

All three strains of the virus grew readily from the beginning. The supernatant fluid from the cultures of the first strain, when inoculated intracerebrally into mice, produced the typical picture of lymphocytic choriomeningitis with spontaneous coarse tremor, spasm and death in six to nine days. At the 25th and 50th subcultures the supernatant fluid was infective in a dilution of 1 : 10,000 (Table I).

TABLE I.—*Titration of Tissue Culture Supernatant Fluid.*

	Dilutions.					
	Undiluted.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
25th subculture	†7, 8, 8, 8	7, 7, 8, 8	7, 8, 9, 9	9, 9, 9, 9	9, 9, 12, 12	9, 9, S, S
50th ,,	7, 7, 8, 8	7, 8, 8, 8	7, 8, 8, 9	8, 9, 9, 9	9, 9, 11, 12	9, 10, 11, S

† = Day of death. S = Survival.

Occasionally mice were sick on the 5th day following inoculation, but always with the symptoms characteristic of typical lymphocytic choriomeningitis. At about the 66th subculture, however, it was noticed that a few of the mice inoculated with supernatant fluid began to show different symptoms of disease on the 5th day after inoculation, as mentioned by Findlay (1939). The clinical picture changed from that of a spastic paralysis to a flaccid paralysis even in mice not showing symptoms till the 6th and 7th days after injection. These symptoms remained constant till the cultivation was stopped at the 270th subculture. The mice inoculated with this variant first appeared slightly

TABLE II.—*Results of Inoculation of Variant into Guinea-pigs.*

Inoculum.	Route of inoculation.	Fever.		Dead or killed on day.	Brain suspension inoculated into mice—symptom produced.
		Day of onset.	Duration in days.		
I. 258th subculture supernatant	0·1 c.c., i.c. + 1·0 c.c. s.c.	3	3	7	Flaccid paralysis.
II. 20 per cent. suspension of brain of I	0·5 c.c., s.c.	—	—	—	—
Reinoculated 6 weeks later with 20 per cent. suspension of 2 M.B.P. of 267th subculture	0·5 c.c., s.c.	—	—	—	—
Reinoculated 3 weeks later with strain "T" 20 per cent. guinea-pig brain suspension	0·2 c.c., s.c.	5	4	10	Spastic paralysis.
III. 258th subculture 1 M.B.P. 20 per cent. suspension of mouse brain	0·2 c.c., i.c.	2	4	9	Flaccid paralysis.
IV. 20 per cent. suspension of brain of guinea-pig III	0·5 c.c., s.c.	10	4	15	Spastic paralysis.
V. 20 per cent. suspension of brain of IV	0·5 c.c., s.c.	5	4	10	Ditto.
VI. 258th subculture 1 M.B.P. 20 per cent. suspension of mouse brain	0·75 c.c., s.c.	—	—	—	—

i.c. = Intracerebral. s.c. = Subcutaneous. M.B.P. = Mouse brain passage.

lethargic in contrast to the hyper-irritability usually seen in mice with typical lymphocytic choriomeningitis, and the coat was ruffled. Then a flaccid paralysis of the hind legs developed, which sometimes was followed by a similar paralysis of the forelegs. Some of the mice did not die till two or three days after the first appearance of signs of disease, whereas with typical lymphocytic choriomeningitis death usually occurred within 24 hours of visible onset of illness.

A rhesus monkey, inoculated intracerebrally with 2·0 c.c. of a 10 per cent. suspension in saline of the brain of a mouse inoculated with the 24th subculture developed a fever on the 6th day, which lasted for three days. There

were no other abnormal signs or symptoms and the monkey made a complete recovery. Blood taken on the 8th day and inoculated intracerebrally into mice produced the typical lymphocytic choriomeningitis.

Two guinea-pigs were inoculated intraperitoneally with 2·5 c.c. of a 15 per cent. suspension in saline of the brain of a mouse inoculated with the 65th subculture. These guinea-pigs showed an elevation of temperature to 104° and 104·5° F. respectively on the 7th day, a peak of 105·8° on the 10th day, followed by a rapid drop in temperature. This was accompanied by tremors and wasting with death on the 13th and 14th days. Mice inoculated intracerebrally with blood and suspensions of brain from these guinea-pigs died from typical lymphocytic choriomeningitis. Inoculation of supernatant fluid from subcultures later than the 70th showed reduction of virulence for guinea-pigs on subcutaneous and intracerebral inoculation.

Six guinea-pigs were inoculated intraperitoneally with 0·75 c.c., and one intracerebrally with 0·2 c.c. of a 20 per cent. suspension in saline of brains of mice with flaccid paralysis following inoculation of the supernatant fluid of the 90th subculture five days previously. None of these guinea-pigs showed any elevation of temperature. Three weeks later the four intraperitoneally inoculated guinea-pigs were reinoculated intraperitoneally with 1·5 c.c. of a 20 per cent. suspension of the brains of mice of the 2nd intracerebral passage of the 95th subculture. No elevation of temperature occurred, and when inoculated three weeks later with River's W.E. strain subcutaneously, all died in the same time as two control guinea-pigs. The remaining two of the original intraperitoneally inoculated guinea-pigs were injected four weeks later with 0·1 c.c. intracerebrally and 0·25 c.c. intraperitoneally of 20 per cent. suspension of brains of mice sick following inoculation of the 200th subculture. They both developed fever on the 5th day, which persisted for six days, then the temperature rapidly fell to 95° F. and the animals died on the 13th day. Blood taken on the 5th day, when the temperature was 105° F., did not show the presence of virus when inoculated intracerebrally in mice.

Although repeated intracerebral passage in mice of the strain producing flaccidity has failed to bring about reversion, it has been possible to reconvert the variant into the original strain by intracerebral passage in guinea-pigs. This has been carried out on numerous occasions following inoculation of either tissue culture supernatant fluid or mouse brain passage material of the supernatant. A typical protocol is given with material from the 258th subculture. The tissue culture supernatant fluid was inoculated intracerebrally, 0·1 c.c., and subcutaneously 1·0 c.c. in a guinea-pig. The temperature rose to 104° F. on the 3rd day ; reached a peak of 105·4° on the 5th day, then fell rapidly to 97°. The animal was thin and weak and was killed on the 7th day. The lungs and other organs appeared healthy. The brain was removed and ground up as a 20 per cent. suspension. When inoculated intracerebrally into six mice it produced flaccid paralysis of the hind legs in three on the 8th day, and weakness in three on the 9th day. None of these mice showed tremors or spasm when spun by the tail. A second mouse brain intracerebral passage resulted in paralysis of the hind legs on the 6th day in all of six mice, and this was maintained in seven more mouse brain passages.

A guinea-pig inoculated subcutaneously with 0·5 c.c. of the 20 per cent.

suspension of the above guinea-pig brain remained healthy with a normal temperature. It was reinoculated six weeks later with 0·5 c.c. of 20 per cent. suspension of 2nd mouse brain passage of the 267th subculture with no ill effects, but died when inoculated subcutaneously three weeks later with 0·2 c.c. of a typical lymphocytic choriomeningitis strain.

The brain of a mouse, showing flaccid paralysis following inoculation with the 258th subculture, was removed and ground as 20 per cent. suspension in saline. 0·2 c.c. of this suspension was inoculated intracerebrally into a guinea-pig. Its temperature rose to 105·4° F. on the 2nd day, remained stationary for four days, then gradually dropped to 99° by the 9th day. The animal became weak and thin on the 7th day and was killed on the 9th day when moribund. All the organs appeared normal to the naked eye. The brain was removed, ground up in a 20 per cent. suspension and inoculated into mice and guinea-pigs. The mice inoculated intracerebrally all developed flaccid paralysis. A guinea-pig inoculated subcutaneously with 0·5 c.c. developed a temperature of 104° F. on the 10th day, which rose to 105·7° on the 12th day. The animal was moribund and killed on the 15th day ; there was extensive consolidation of all lobes of the lung. Blood taken on the 12th day produced flaccid paralysis when inoculated intracerebrally in mice. The brain when inoculated intracerebrally into mice produced typical spasm of lymphocytic choriomeningitis, which was repeated on intracerebral passage in mice. A guinea-pig was inoculated subcutaneously with 0·5 c.c. of this same guinea-pig brain suspension. It had a temperature of 106° F. on the 6th and 7th days, which fell gradually till the 10th day, when the animal was killed as it was weak and thin. The brain of this guinea-pig also produced typical lymphocytic choriomeningitis when inoculated intracerebrally in mice.

0·75 c.c. of the 258th subculture, 1st mouse brain passage, produced no elevation of temperature when inoculated subcutaneously in a guinea-pig.

DISCUSSION.

The growth of viruses in large quantities in tissue culture provides suitable material for vaccines and for physical and chemical studies of yellow fever, vaccinia and other viruses. Some of these viruses during repeated passage in tissue culture, or on the chorio-allantoic membrane of the developing chick embryo, have become sufficiently attenuated to be of use, with less chance of reaction, for human immunization as in vaccinia and yellow fever. Other viruses, as pseudo-rabies (Glover, 1939), have produced variants which cause symptoms different from those of the original strain when inoculated into animals. Still other viruses have shown no change. Thus, a strain of fowl pox originally obtained from a sparrow and virulent only for canaries retained its full virulence for more than 80 subcultures in chick embryo-Tyrode medium. The observations here recorded show that the virus of lymphocytic choriomeningitis grows readily in a simple medium of chick embryo tissue and serum Tyrode's solution without any diminution in its virulence for mice after numerous subcultures, though in one instance, at the 66th passage, it became

attenuated for guinea-pigs. No apparent attenuation of the virus has taken place despite the change in the clinical picture in mice, but experiments are now being carried out to see whether the tissue culture virus differs antigenically from that passaged in mice and guinea-pigs. Since the virus is grown in tissue culture and largely freed from extraneous antigen, it should possibly prove a better immunizing agent than virus obtained directly from animal tissue. Traub (1938), for instance, found that formalinized virus in guinea-pig tissues would immunize guinea-pigs, but had no action in mice owing to the presence of foreign antigens.

Recently Smadel, Baird and Wall (1939) and Smadel, Wall and Baird (1940) have isolated a soluble antigen from the virus of lymphocytic choriomeningitis, and have found that it gives more satisfactory results than active virus in complement-fixation and precipitin tests. Experiments with our tissue culture virus suggest that it should be a satisfactory source of antigen, free of guinea-pig and mouse tissue, for these immunological tests.

It is difficult to account for the production of a variant of the lymphocytic choriomeningitis virus which, without loss of virulence, produces entirely new symptoms in mice, since after passage in guinea-pigs the virus again produces the original symptoms in mice, but does not appear to be stable. It is hoped that continued passage of other strains will shed further light on this variation, though Anderson (1939) has been unable to repeat an attenuation of a neurotropic strain of herpes simplex which occurred following numerous passages on the chorio-allantois of chick embryos.

SUMMARY.

Three strains of the virus of lymphocytic choriomeningitis have been grown in a simple medium of minced chick embryo and serum Tyrode's solution and carried for 66, 38 and 35 subcultures respectively without loss of virulence for guinea-pigs or mice.

The first strain produced a new non-stable variant, probably a *Dauermodifikation*, after approximately 66 passages. No further change occurred in 204 further passages. The variant produced new symptoms in mice and showed reduction in pathogenicity for guinea-pigs. Rapid passage in guinea-pigs caused the virus to revert to its original condition.

The possibility of using tissue culture strains of lymphocytic choriomeningitis virus for vaccine production and as a source of antigen for complement-fixation and precipitin tests is discussed.

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